POLYPEPTIDE DEGRADATION BY DIPEPTIDYL AMINOPEPTIDASE I (CATHEPSIN C) AND RELATED PEPTIDASES

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1. INTRODUCTION

Whereas the reader will undoubtedly recognize the term "cathepsin C," few will recognize the term "dipeptidyl aminopeptidase I." To consider cathepsin C a dipeptidyl aminopeptidase may actually seem incompatible with respect to the restricted catalytic properties that have been reported for this enzyme. Following the discovery of cathepsin C in swine kidney by Gutmann and Fruton (1948), a series of classic papers concerned with the properties of this enzyme, published by Fruton and his associates, led to the conclusion that the substrate specificity of cathepsin C was too severely restricted to permit its meaningful contribution to tissue proteolysis (for references, see Izumiya and Fruton 1956; Fruton 1957; Metrione et al. 1966). Studies conducted by others on peptide derivatives and polypeptides led to the same conclusion (Planta and Gruber 1961; Planta et al. 1964). More recent papers from Fruton's laboratory focused on the polymerase activity of cathepsin C. In those papers, cathepsin C was called "dipeptidyl transferase" (Voynick and Fruton 1968; Heinrich and Fruton 1968). The term "aminopeptidase," on the other hand, denotes a hydrolytic activity and, generally speaking, suggests a relatively broad substrate specificity. The studies described in this report are intended to show that cathepsin C does, indeed, have a broad substrate specificity, and that it should, like some other newly-found tissue peptidases mentioned here, be classified as a dipeptidyl aminopeptidase. As the term implies, these enzymes degrade peptides through a process of fragmentation that begins with an attack at the unsubstituted NH_2 terminus of the substrate, and catalyzes the successive removal of amino acids as dipeptides.

The text of this report deals with the specificities and properties

of dipeptidyl aminopeptidase I (cathepsin C) from rat liver, bovine spleen, and rat and bovine pituitary glands; dipeptidyl aminopeptidases II and III of the anterior pituitary gland; and dipeptidyl aminopeptidase IV ("Gly-Pro-β-naphthylamidase") of kidney and liver. The distribution of these enzymes in the tissues, their subcellular localization, and their possible physiological significance will also be considered.

2. DIPEPTIDYL AMINOPEPTIDASE I (CATHEPSIN C)

2.1. Background

The chloride requirement and broad substrate specificity of dipeptidyl aminopeptidase I were first recognized in preparations derived from the bovine anterior pituitary gland. The original reports (McDonald et al. 1965, 1966a) described the pituitary enzyme as a chloride-activated, sulfhydryl-dependent dipeptidyl arylamidase that catalyzed the cleavage of Ser-Tyr from Ser-Tyr-β-naphthylamide (a fluorogenic model of the NH_2 terminus of adrenocorticotropic hormone) and from the NH_2-terminal decapeptide of adrenocorticotropic hormone (abbreviated ACTH). The pituitary enzyme had an optimum at about pH 4.0, that increased with purification, and its action was restricted to substrates having an unsubstituted NH_2 terminus. At about pH 6.5 it was possible to obtain a 4-to 6-fold enhancement of the rate of free β-naphthylamine formation from Ser-Tyr-β-naphthylamide by adding Gly-Phe-NH_2 or puromycin (a p-methoxy-L-phenylalanyl aminonucleoside) to reaction mixtures (McDonald et al. 1965). Chloride and sulfhydryl requirements were also demonstrated for the enhancement phenomenon. It was postulated that the added Gly-Phe-NH_2

and puromycin served as acceptors in a transferase reaction catalyzed by the pituitary "dipeptidyl arylamidase I." It was not possible, at that time, to attribute the activities of the pituitary enzyme to cathepsin C. We, as well as others (Vanha-Perttula and Hopsu 1965), had not been able to demonstrate cathepsin C activity in pituitary extracts using the traditional Gly-Phe-NH_2 substrate. Furthermore, the chloride requirement found for the pituitary enzyme had never been reported for cathepsin C.

To help establish the identity of the pituitary enzyme, it became necessary to test spleen cathepsin C for a chloride requirement, and to compare its substrate specificity with that of the pituitary enzyme. As reported (McDonald et al. 1966b), partially purified preparations of cathepsin C from both bovine spleen and rat spleen were found to have an apparently absolute chloride requirement. Through a personal communication, it was learned that Gorter and Gruber, at Groningen University, had also recently observed the chloride requirement of bovine spleen cathepsin C. For many years the activity of bovine spleen cathepsin C was studied, fortuitously, in chloride-or bromide-containing reaction mixtures (Izumiya and Fruton 1956; Planta and Gruber 1963; Vanha-Perttula et al. 1965), primarily because dipeptide amide substrates were prepared and used as hydrochloride salts. On the other hand, attempts to assess the activity of the enzyme on peptides and proteins may have been jeopardized by chloride deficiencies unless a chloride-containing sulfhydryl activator was used, i.e., cysteine hydrochloride or 2-mercaptoethylamine hydrochloride. Chloride deficiencies may account for the negative results obtained on glucagon (Planta and Gruber 1961; Planta et al. 1964), and

the inability of others to detect glucagon-degrading activity in extracts of liver lysosomes (Kenny 1956, 1958).

Since the relative effect of the different halides on spleen dipeptidyl aminopeptidase I, including the replacement effect of nitrate, resembled properties already reported for the pituitary enzyme, the possibility arose that these enzymes had a common identity. Based on its ability to degrade peptides such as ACTH and alanine peptides, the pituitary enzyme was renamed "dipeptidyl aminopeptidase I" (McDonald et al. 1968a), replacing the term "dipeptidyl arylamidase I." However, before any conclusions could be reached regarding the possible common identity for the pituitary and spleen enzymes, it became essential to examine the substrate specificity of highly purified bovine spleen cathepsin C. In addition, since liver preparations exhibited a chloride- and sulfhydryl-dependent hydrolysis of His-Ser-β-naphthylamide, a fluorogenic model of the NH_2 terminus of glucagon (McDonald et al. 1966b, 1969a), cathepsin C was therefore purified from rat liver, as well as bovine spleen, for the purpose of investigating the possible identity of the so-called "glucagon-degrading enzyme" of liver -- a chloride- and sulfhydryl-dependent enzyme that was thought to be distinct from cathepsin C (Kakiuchi and Tomizawa 1964).

The studies described in this report were conducted with cathepsin C purified according to Metrione, Neves, and Fruton (1966). This method yields a highly purified bovine spleen cathepsin C with a specific activity about nine times greater than that achieved by the method of de la Haba et al.(1959). Even higher specific activities were achieved when rat liver was used as a source of cathepsin C (McDonald et al. 1969b). Cathepsin C from bovine spleen and rat liver was found to have many properties common to the pituitary enzyme. In addition to other similarities,

these enzymes showed the same substrate specificity on polypeptide substrates, in particular, the ability to catalyze the removal of dipeptides in sequence. Considering the nature of their degradative activity and the evidence for their common identity, cathepsin C from bovine spleen and rat liver are herein referred to as "dipeptidyl aminopeptidase I."

2.2. Degradation of Polypeptides

The degradation of peptide hormones by dipeptidyl aminopeptidase I has yielded new information about the substrate specificity of this enzyme (McDonald and Ellis 1968; McDonald et al. 1969a, 1969b).

The degradation of β-corticotropin by rat liver dipeptidyl aminopeptidase I (Fig. 1) is representative of the results obtained with the spleen and pituitary enzymes. These findings revealed a surprisingly broad substrate specificity for the enzyme. Although the extent of ACTH degradation (25%) was less than what was subsequently demonstrated with other hormone substrates, the results of the time course analysis (Fig. 1) provided a reasonably unambiguous, visual illustration of the sequential release of dipeptides by dipeptidyl aminopeptidase I. Upon the release of Ser-Tyr from ACTH, which was anticipated (Planta and Gruber 1961; McDonald et al. 1965), the next eight residues were liberated sequentially in pairs: Ser-Met, Glu-His, Phe-Arg, and Trp-Gly. The apparent low yield of Trp-Gly was actually the result of a weak ninhydrin color reaction, as demonstrated by the Trp-Gly standard. The rat liver and bovine spleen enzymes exhibited the same degradative activity on both native porcine ACTH (39 residues) and synthetic β^{1-24}-corticotropin. Comparable bonds were cleaved in a related, small fragment, Phe-Arg-Trp-Gly-OMe. An NH_2-terminal D-serine,

as in D-Ser^{1}, Lys^{17,18}-β^{1-18}-corticotropinamide, which renders the hormone resistant to attack by leucine aminopeptidase (Boissonnas et al. 1966), did not prevent the degradation of the hormone by dipeptidyl aminopeptidase I. D-Ser-L-Tyr was cleaved at 20 to 25% of the rate of L-Ser-L-Tyr (McDonald et al. 1969b). By comparison, α-melanophore-stimulating hormone, which has an NH_2-terminal sequence like that of β-corticotropin, but with an N^{α}-acetyl substituent, was not hydrolyzed. These findings are consistent with earlier studies showing that blocked dipeptide derivatives are not hydrolyzed (Wiggans et al. 1954).

An analysis of the specific bonds cleaved in ACTH revealed some unexpected substrate specificity characteristics of the enzyme. Ser-Met was readily removed in contrast to results obtained with Gly-Met-NH_2 (Tallan et al. 1952). The cleavage of the penultimate histidyl and arginyl bonds was of special interest in view of earlier reports that the hydrolytic activity of the enzyme was virtually restricted to the carbonyl side of residues having a hydrophobic side chain (Izumiya and Fruton 1956; Planta et al. 1964; Voynick and Fruton 1968). As will be seen, arginyl and lysyl bonds were hydrolyzed more rapidly than any other; however, the properties of the NH_2-terminal residue can greatly modify these rates. On the other hand, it was not surprising to find that the degradation of ACTH failed to proceed beyond the tenth residue. At that point an NH_2-terminal lysine (residue 11) arose. It had already been demonstrated that dipeptidyl aminopeptidase I has no action on dipeptide amides with an NH_2-terminal lysine, i.e., Lys-Phe-NH_2 and Lys-Tyr-NH_2 (Izumiya and Fruton 1956). As will be seen, the penultimate prolyl residue (at position 12) constituted an additional obstruction.

The degradative activity of dipeptidyl aminopeptidase I was further

demonstrated using several other polypeptide hormones as substrates. These included glucagon and secretin (McDonald and Ellis 1968; McDonald et al. 1969a); the B chain of oxidized bovine insulin and the des-Phe B chain (McDonald et al. 1969b; Callahan et al. 1969); $Asp^1(NH_2)$-angiotensin II (CIBA hypertensin) and Trp-Met-Asp-Phe-NH_2, the physiologically active, COOH terminus of gastrin (McDonald et al. 1969b); and synthetic, human Ile^5-angiotensin II (unpublished). The degradation observed in these substrates is summarized in Fig. 2. The "arrows" shown along the top of each sequence indicate the points of cleavage, and the extent to which each hormone was degraded.

The biological activity of each of the hormonally active peptides shown in Table 2 was lost with the cleavage of the first dipeptide. This was shown to be the case for glucagon (McDonald et al. 1969a), and would be predicted for the other hormones on the basis of the known structural requirements of ACTH (White 1955; Schwyzer 1963), the gastrin tetrapeptide (Tracy and Gregory 1964), and, according to Ondetti (McDonald et al. 1969a), secretin. In the case of angiotensin II and angiotensin II amide, wherein the loss of the NH_2-terminal aspartyl, or asparaginyl, residue results in a 50% loss of vasopressor activity, the loss of the NH_2-terminal dipeptide should destroy all the pressor activity of the hormone (Schwyzer and Turrian 1960).

As seen in Fig. 2, dipeptidyl aminopeptidase I catalyzed an extensive breakdown of all the peptides tested. In the pH range of 5.0 to 5.5, it was possible to degrade these peptides over a 1 to 2 hour time course using a molar ratio of enzyme to substrate between 10^{-5} to 10^{-3}. The higher ratios were required for the maximal digestion of substrates such as glucagon, secretin, and the B chain of oxidized insulin; the lower ratios

for ACTH, angiotensin II, and the gastrin tetrapeptide. In every instance, only dipeptides were liberated, in sequence, indicating the absence of any intrinsic or contaminating endopeptidase activity. NH_2-Terminal analyses conducted on the digest during the degradation of glucagon failed to show points of cleavage other than those attributable to the release of dipeptides. While it is obvious that a wide variety of bonds were cleaved, an examination of Fig. 2 shows that the enzyme has some clearly defined limitations. Dipeptidyl aminopeptidase I was unable to remove the Arg-Arg dipeptide that arises from Residues 17 and 18 of glucagon. Neither was it able to hydrolyze Arg-Arg-β-naphthylamide. The results obtained with secretin show that Ser-Arg (Residues 11 and 12), Leu-Arg (Residues 13 and 14), and Ala-Arg (Residues 17 and 18) were readily cleaved; however, the emergence of Arg-Leu (Residues 21 and 22) prevented further degradation of secretin. It seems reasonable to assume that the leucyl residue should otherwise have been hydrolyzed since Glu-Leu (in secretin), Tyr-Leu (in glucagon), Gly-Leu (in insulin A chain), and His-Leu (in insulin B chain) were easily hydrolyzed. It was concluded, therefore, that the degradative activity of dipeptidyl aminopeptidase I can be blocked, not only by the appearance of a terminal lysine residue but also by the emergence of an NH_2-terminal arginine. On the other hand, histidine residues, either terminal (as in glucagon and secretin), or penultimate (as arises in ACTH and the B chain of insulin), are no obstacle.

Fig. 2 also contains examples of the resistance of proline residues. The COOH-terminal tetrapeptide of the B chain of insulin (Thr-Pro-Lys-Ala) was not hydrolyzed, nor was Gly-Pro-β-naphthylamide. Earlier studies by Planta et al.(1964) showed that a tripeptide, Thr-Pro-Lys, was also resistant to hydrolysis; however, as will be seen, it is more meaningful to

evaluate the substrate specificity of dipeptidyl aminopeptidase I on peptides having at least four residues. These results show that the continued degradation of a polypeptide can be blocked by the emergence of a penultimate prolyl residue. Furthermore, dipeptidyl aminopeptidase I was unable to hydrolyze Val-His-Pro-Phe and Ile-His-Pro-Phe, the residual COOH-terminal tetrapeptides arising from the breakdown of the two angiotensin II substrates. Referring ahead to Fig. 15, this resistance is evident from the products of Asp^1 (NH_2)-angiotensin II degradation at pH 8.0 by dipeptidyl aminopeptidase III, the results of which are identical to those obtained with dipeptidyl aminopeptidase I at pH 5.5. Proline-containing tripeptides such as Gly-Phe-Pro and Val-Tyr-Pro are also resistant (Planta et al. 1964). Thus, it appears that dipeptidyl aminopeptidase I is unable to cleave dipeptides from the imino nitrogen of proline. This restriction is reminiscent of similar restrictions reported for chymotrypsin (Shepherd et al. 1956) and leucine aminopeptidase (Frater et al. 1965). The inability of dipeptidyl aminopeptidase I to cleave dipeptides from proline is consistent with the inability of this enzyme to polmerize dipeptide amides and esters containing NH_2-terminal proline (Wiggans et al. 1954). In summary, a prolyl residue constitutes an impasse for dipeptidyl aminopeptidase I if it occurs in a peptide substrate at any position other than at the initial NH_2 terminus.

A variety of non-physiological peptides were also used to help delineate the substrate specificity of dipeptidyl aminopeptidase I. For example, Val-Leu-Ser-Glu-Gly was degraded to Val-Leu, Ser-Glu, and glycine. Polyalanine (molecular weight 2000 to 5000), Ala_6 and Ala_4 were degraded to Ala_2 from their NH_2 termini. However, in the case of Ala_5, the products were Ala_2 and Ala_3. Ala_3 was not hydrolyzed by dipeptidyl

aminopeptidase I. On the other hand, derivatives of Ala_2 were easily hydrolyzed, i.e., the amide, the β-naphthylamide, and the methyl ester. Phe_4 and Gly_4 were rapidly cleaved to dipeptides, whereas the tripeptides, Phe_3 and Gly_3, were hydrolyzed at relatively slow rates. It thus appears that tripeptides may be relatively poor substrates for dipeptidyl aminopeptidase I. Glu_4 was hydrolyzed slowly, and Lys_4 not at all.

Table I presents a summary of all the NH_2-terminal dipeptides that were found to be susceptible to cleavage from model polypeptides. The liberation of many of these dipeptides was also demonstrated with dipeptide derivatives. However, except for a few tests that were conducted with only β-naphthylamides, all the findings included in Table I were derived from the use of peptide substrates. An examination of the dipeptides included in Table I shows that all the commonly occurring NH_2-terminal residues have been evaluated except for cysteine and isoleucine. Among the COOH-terminal residues (which represent the penultimate residues in the peptide substrate), aspartic acid, cysteine, isoleucine, and threonine are not represented. Although no cysteine-containing NH_2-terminal dipeptides were tested, it was found that a cysteic acid residue (terminal or penultimate) was no obstacle to the action of dipeptidyl aminopeptidase I. Only a small percentage (about 16%) of the total number of possible dipeptides are represented in Table I. Since multiple combinations are shown for most of the NH_2-terminal and COOH-terminal residues tested, some fairly reliable predictions should be possible regarding the susceptibility of untested dipeptides.

In summary, it appears that the degradation of a peptide by dipeptidyl aminopeptidase I can be completely blocked by (a) an NH_2-terminal arginyl or lysyl residue, (b) a penultimate prolyl residue, or (c) any

dipeptide that is bonded to the imino nitrogen of a prolyl residue. The only exception encountered was on Gly-Trp-β-naphthylamide (McDonald et al. 1969b), a relatively insoluble derivative. However, since Gly-Trp-NH_2 is hydrolyzed (Wurz et al. 1962), the meaning of this negative result is of questionable significance. In spite of its limitations, dipeptidyl aminopeptidase I can exhibit potent degradative activity. For example, the total number of bonds cleaved (thirteen) in the B chain of oxidized insulin is equal to the net effect of trypsin, chymotrypsin, and pepsin, each acting at its own pH optimum.

2.3. Halide Requirement

The halide requirement exhibited by rat liver and beef spleen dipeptidyl aminopeptidase I is virtually absolute for the hydrolysis of dipeptide β-naphthylamides (Fig. 3). On the other hand, it is not known whether the enzyme is equally dependent on halide ions for the hydrolysis of polypeptides. No effort was made to remove contaminating salts from the peptide substrate. Even so, it was relatively easy to demonstrate a very pronounced chloride activation on polypeptide substrates. Fig. 1 includes an example of the amount of ACTH hydrolyzed in the absence of added chloride. Without added chloride ions, a small amount of Ser-Tyr and a trace of Ser-Met were cleaved from ACTH after 30 minutes of incubation. However, with added chloride (16 mM) a good yield of the first four dipeptides was obtained in 30 minutes. A very pronounced chloride requirement was also easily demonstrated for the hydrolysis of glucagon by rat liver dipeptidyl aminopeptidase I. When chloride was withheld from a reaction mixture which otherwise showed an extensive degradation of glucagon, the amount of His-Ser cleaved from the NH_2 terminus of glucagon

was barely discernible.

The low K_m values (0.1-0.2 mM at pH 6.0) exhibited on the β-naphthylamide substrates, together with the low concentrations of enzyme required for the fluorometric assay technique, made it relatively simple to minimize halide contaminants, and maximize the halide sensitivity of the system. This was undoubtedly a critical factor leading to the initial detection of the chloride requirement for the pituitary enzyme (McDonald et al. 1965). By comparison, the dipeptide amides are normally used at substrate concentrations that are about 200 times greater. The direct, continuous recording, fluorometric assay method (McDonald et al. 1969b) therefore seems to offer an ideal system for kinetic studies designed to reveal the nature of the chloride effect.

In Fig. 3, the rate response to halide concentration is shown for the hydrolysis of Gly-Phe-β-naphthylamide by rat liver dipeptidyl aminopeptidase I. The response to chloride, bromide, and iodide was maximal at about 5 mM where the relative effects were $Cl^- > Br^- > I^- > F^-$. Nitrate and nitrite showed a limited ability to substitute for the halides. At limiting concentrations (< 2 mM) of these halides, Br^- and I^- seemed more effective than Cl^-. No activation was detected with acetate, citrate, phosphate, sulfate, or cacodylate. The chloride activation was also evident on many other dipeptide β-naphthylamide substrates, including basic ones such as Gly-Arg-β-naphthylamide and Pro-Arg-β-naphthylamide.

Although the mechanism of halide action is still obscure, it can be assumed that the halide ion either makes some essential charge contribution at the active center complex, which is postulated to be an acyl (dipeptidyl)-thiol ester (Voynick and Fruton 1968), or it may be involved in a multisite interaction on the enzyme whereby the halide behaves as

an "effector" that aids the substrate in transforming the enzyme into a catalytically active state. In any event, whatever the nature of the chloride effect, the broad substrate specificity exhibited by the enzyme shows that the reaction mechanism does not require the presence of a hydrophobic side chain on the residue containing the activated carbonyl group (Voynick and Fruton 1968).

2.4. Kinetics

Little information is available concerning the kinetics of dipeptide cleavage from polypeptide substrates by dipeptidyl aminopeptidase I. However, there is some indication that dipeptide β-naphthylamides may be used as substrates to gain information regarding the kinetics of polypeptide hydrolysis. For example, the kinetics of His-Ser-β-naphthylamide hydrolysis at pH 6.0 by the rat liver enzyme was found to resemble the kinetics of His-Ser cleavage from glucagon. His-Ser-β-naphthylamide had a K_m of 0.022 mM ± 0.002 S.E., and glucagon 0.027 mM ± 0.01 S.E. Although these values are very similar, they are exceptionally low compared to most other dipeptide β-naphthylamides (Table 2). Obviously, the K_m value found for glucagon applies only to the removal of the first dipeptide (His-Ser).

The maximum turnover rate constant, k_{cat}, for the first dipeptide of glucagon, calculated from V_{max}, was found to be about 81 sec^{-1}. The value of $k_{cat}/K_{m\ app}$ was thereby estimated to be about 3,000 mM^{-1} sec^{-1}, reflecting a high degree of preference for the glucagon substrate compared to many of the dipeptide amides and esters that have been tested (Voynick and Fruton 1968; McDonald et al. 1969b). Similarly, the rat liver enzyme exhibited a preference for His-Ser-β-naphthylamide that was comparable to

that observed on glucagon. For example, the k_{cat} for His-Ser-β-naphthylamide was about 116 sec^{-1}, making $k_{cat}/K_{m\ app}$ equal to about 5,300 mM^{-1} sec^{-1}. The calculation of k_{cat} values for the rat liver enzyme was based on a molecular weight of 200,000. This value, which was estimated from the filtration rate of the enzyme on a calibrated column of Sephadex G-200 (Andrews 1965), compares with the molecular weight of the bovine spleen enzyme calculated from sedimentation equilibrium measurements (Planta and Gruber 1964; Metrione et al. 1966).

Glucagon and Gly-Phe-NH_2 were both found to be competitive inhibitors of His-Ser-β-naphthylamide hydrolysis by rat liver dipeptidyl aminopeptidase I. Glucagon, however, with a K_i of 0.08 mM, was by far a better inhibitor than Gly-Phe-NH_2 with a K_i of about 4.7 mM. It was therefore evident that glucagon, and probably also His-Ser-β-naphthylamide, were bound to the enzyme more tenaciously than was Gly-Phe-NH_2.

In a reaction mixture containing 1.5 mM ACTH (β^{1-24}-corticotropin) at pH 5.0, rat liver dipeptidyl aminopeptidase I cleaved the first dipeptide, Ser-Tyr, at a (near initial) velocity of about 24 μmoles min^{-1} mg^{-1} enzyme protein. By the time 84% of the Ser-Tyr had been cleaved, 20% of the second dipeptide, Ser-Met, had been cleaved. In a similar reaction mixture, containing D-Ser^1-ACTH (D-Ser^1, $Lys^{17,18}$-β^{1-18}-corticotropinamide), D-Ser-L-Tyr was cleaved at a rate of only 5 μmoles min^{-1} mg^{-1}. A relatively far greater affinity was exhibited for the second dipeptide, Ser-Met, as indicated by the finding that 20% of the Ser-Met had been cleaved by the time D-Ser-L-Tyr reached 23%. As seen in Table 2, the first four dipeptides cleaved from ACTH were also studied as β-naphthylamide substrates, at pH 6.0. Ser-Tyr was cleaved at a rate of 28 (compared with 24 for the hormone), Ser-Met at 100, Glu-His at 33, and Phe-Arg at 23 μmoles min^{-1} mg^{-1} enzyme.

Based on a limited number of kinetic studies comparing polypeptides and dipeptide derivatives as substrates for dipeptidyl aminopeptidase I, it appears that the bulky β-naphthylamide substrates, in contrast with esters and amides, may yield results that are more analogous to the action of the enzyme on polypeptides. In Table 2, the rates of hydrolysis are shown for a variety of dipeptide β-naphthylamides. Hormones are cited wherein the same dipeptide has been shown to be susceptible to attack by the enzyme. Exceptionally high rates of hydrolysis were observed on substrates containing a penultimate arginyl residue. Similarly, among a group of esters, Gly-Lys-OMe was hydrolyzed most rapidly.

In contrast to reports describing its preferential attack on hydrophobic residues (Izumiya and Fruton 1956; Planta et al. 1964; Voynick and Fruton 1968), dipeptidyl aminopeptidase I from both rat liver and bovine spleen, catalyzed the cleavage of penultimate arginyl and lysyl bonds at rates that were, in general, many orders of magnitude higher than the rates observed on most other penultimate residues. Comparative studies with amides and methyl esters have revealed that β-naphthylamides give the lowest K_m values (0.1 to 0.2 mM at pH 6.0) with both the rat liver and bovine spleen enzymes. By comparison, K_m values for the esters and amides of comparable dipeptides are about 10 and 100 times higher, respectively (McDonald et al. 1969b).

2.5. Effect of pH

Fruton and Mycek (1956) showed that bovine spleen dipeptidyl aminopeptidase I has a pH optimum close to 6.0 for the hydrolysis of Gly-Tyr-NH_2. Although the hydrolytic activity of the enzyme is generally measured at pH 6.0 (Metrione et al. 1966), a small amount of polymerization of

Gly-Arg-β-naphthylamide was detected at pH 6 using analytical methods already described (McDonald et al. 1969b). For this reason, polypeptide digests were maintained at pH 5.0, with a volatile buffer-activator mixture (pyridine-HCl-acetic acid-2-mercaptoethanol). We have thus far not attempted to demonstrate transpeptidation using peptides as substrates; however, studies concerned with the effect of pH on the hydrolysis of peptides suggest that little, if any, transpeptidation occurs. For example, the pH optimum for the cleavage of His-Ser from glucagon was unusually high. The accumulation of free His-Ser from glucagon was optimal at about pH 6.5. Even at pH 7.5, where polymerization (of dipeptide amides) usually predominates (Nilsson and Fruton 1964), His-Ser accumulated at a rate that was 50% of the optimal rate at pH 6.5. Such a pH response approaches that obtained on Pro-Phe-NH_2, a substrate that is not susceptible to transpeptidation (Izumiya and Fruton 1956), and is cleaved optimally at pH 7.0 (Fruton and Mycek 1956). Dipeptidyl aminopeptidase I also exhibited a pH 7.0 optimum on Pro-Arg-β-naphthylamide (McDonald et al. 1969b). In comparison with the pH optimum found on glucagon, a much lower and more discrete pH optimum of 5.2 was found on His-Ser-β-naphthylamide. At pH 5.2 the rate of His-Ser liberation from the β-naphthylamide was about twice that found for glucagon; at pH 6.5 the opposite was found; and at pH 6.0 the rates were the same.

Using $Asp^1(NH_2)$-angiotensin II as a substrate for the rat liver enzyme, the first dipeptide (Asn-Arg) was cleaved optimally at pH 5.5. At pH 7.0 the rate was reduced 50%. The second dipeptide (Val-Tyr) was cleaved optimally at about pH 6.5. At pH 5.5 Val-Tyr accumulated with a noticeable lag. For example, after 70% of the Asn-Arg had accumulated, about 40% of the Val-Tyr was free. However, at pH 7.0 Val-Tyr accumulated

at a rate approaching that found for the first dipeptide (Asn-Arg). On the other hand, the cleavage of Asp-Arg from synthetic human angiotensin occurred optimally at pH 4.5 or lower.

Although the optimal pH may vary for each dipeptide cleaved from a polypeptide by dipeptidyl aminopeptidase I, it has thus far been possible to carry polypeptide degradation to completion (to the extent possible) between pH 5.0 and 5.5.

2.6. Activators and Inhibitors

The sulfhydryl requirement of beef spleen dipeptidyl aminopeptidase I and the effects of various inhibitors were studied by Fruton and Mycek (1956) using dipeptide amide substrates. Similarly, in our studies, Hg^{++}, p-chloromercuriphenyl sulfonate, and oxidized glutathione exhibited reversible inhibition. If the concentrated enzyme were first activated with a sulfhydryl compound and the activator diluted out (under a nitrogen atmosphere), it was possible to obtain a 73% inhibition of Gly-Arg-β-naphthylamide hydrolysis by incorporating oxytocin, a disulfide hormone, at 4×10^{-6}M. When dipeptidyl aminopeptidase I was first found to exhibit extraordinary rates of hydrolysis on arginyl and lysyl bonds, pancreatic trypsin inhibitor was tested and found to be ineffective, even at high levels. Preincubation of the enzyme in 1 mM diisopropylfluorophosphate failed to inhibit the degradation of angiotensin II, a result that agreed with an earlier study using a Gly-Tyr-NH_2 substrate (Fruton and Mycek 1966).

2.7. Subcellular Localization

2.7.1. Biochemical studies

Early evidence for the lysosomal localization of dipeptidyl aminopeptidase I

was obtained by conventional methods of differential centrifugation. The activity was first found in mitochondrial fractions by Finkenstaedt (1957). The probability of a lysosomal localization was further strengthened by the work of Bouma and Gruber (1966), who showed that the liver enzyme was closely associated with acid phosphatase after density-equilibrium centrifugation of a lysosome-rich fraction that was prepared according to de Duve et al. (1955). A similar technique was used for the subcellular fractionation of rat pituitary glands (McDonald et al. 1968b). Both dipeptidyl aminopeptidase I (assayed on Ser-Tyr-β-naphthylamide) and acid phosphatase were located in the same region of the sucrose gradient. Pellets prepared from each gradient fraction were examined by electron microscopy. The lysosomes were shown to be concentrated at an isopycnic point that was coincident with the maximum activities of dipeptidyl aminopeptidase I and acid phosphatase. Dipeptidyl aminopeptidase II, assayed on Lys-Ala-β-naphthylamide, was also located here. A high latency was exhibited by both dipeptidyl aminopeptidase I and II. Prior to the addition of Triton X-100, about 95% of these activities was masked and sedimentable. In contrast to the preceding studies, Shibko and Tappel (1965) were unable to locate dipeptidyl aminopeptidase I in kidney lysosomes, nor did they demonstrate its presence in any other subcellular fraction. The inability of the latter authors to localize the enzyme is presently inexplicable, but may be attributable to the insensitivity of their assay method, which involved the use of ninhydrin for the detection of Gly-Phe-NH_2 hydrolysis. Furthermore, it appears that their assay system was deficient in Cl^-.

2.7.2. Electron microscopy

Recent unpublished studies have demonstrated the presence of dipeptidyl

aminopeptidase I in the lysosomes of rat liver and anterior pituitary by electron microscopy, using both histochemical and immunohistochemical techniques.

Pro-Arg-4-methoxy-β-naphthylamide was adopted as the histochemical substrate for the following reasons. It is hydrolyzed specifically by dipeptidyl aminopeptidase I at pH 5.5 at a rate comparable to that found on Pro-Arg-β-naphthylamide (Table 2); the NH_2-terminal proline makes the substrate resistant to the action of aminopeptidases, and prevents its polymerization by dipeptidyl aminopeptidase I; the substrate is readily soluble; and the reaction product, 4-methoxy-β-naphthylamine, which is less soluble than β-naphthylamine, can be reacted simultaneously with a diazonium salt such as fast blue B or hexazotized pararosaniline to furnish a stable azo dye that chelates metals to provide the necessary color intensity or electron opacity (Rutenburg et al. 1968; Smith 1969b). Such a technique was used to demonstrate dipeptidyl aminopeptidase I at pH 5.5 in lysosomes and autophagic bodies in the livers of glucagon-treated rats (Fig. 4), and in the lysosomes of livers taken from control animals (Fig. 5). Glucagon was administered to rats in accordance with Deter and de Duve (1967) for the purpose of inducing an autophagic process within hepatocytes. The enzyme was also shown to have a lysosomal distribution in the gonadotrophic cells of the rat pituitary gland (Fig. 11).

Dipeptidyl aminopeptidase I was also detected immunohistochemically using a technique developed by Nakane and Pierce (1967) for the localization of antigens. Sections of rat liver were reacted with a rabbit antiserum prepared against purified rat liver dipeptidyl aminopeptidase I. The sites of bound rabbit γ-globulin were detected with a goat anti-rabbit γ-globulin labeled with horseradish peroxidase. With such a technique it was possible

to show that the discrete localization of dipeptidyl aminopeptidase I seen by light microscopy (Fig 6) was attributable to a lysosomal localization as seen by electron microscopy (Fig. 7).

The immunochemical results verified those obtained histochemically with Pro-Arg-4-methoxy-β-naphthylamide, and demonstrated that dipeptidyl aminopeptidase I is exclusively lysosomal in the liver and anterior pituitary gland of the rat.

3. DIPEPTIDYL AMINOPEPTIDASE II

Another lysosomal enzyme has been discovered that has the properties of a "dipeptidyl aminopeptidase" (McDonald et al. 1968a; 1968b). This enzyme was first detected in extracts of bovine anterior pituitary glands, but has since been shown to have a wide distribution. As will be seen (Table 7), the thyroid gland appeared to be the richest source, and here, too, the enzyme had a lysosomal distribution.

3.1. Specificities and Properties

Dipeptidyl aminopeptidase II was initially detected in pituitary extracts by its ability to hydrolyze Lys-Ala-β-naphthylamide at pH 5.5 (K_m=0.011 mM at 37°C). This substrate is resistant to hydrolysis by dipeptidyl aminopeptidase I. The reaction products were Lys-Ala and free β-naphthylamine. The responsible enzyme, originally termed "dipeptidyl arylamidase II" (McDonald et al. 1968b), acted on a limited number of dipeptide β-naphthylamide substrates having unsubstituted NH_2 termini.

The enzyme was purified more than 1,000-fold over a pH 5.5 aqueous extract of bovine anterior pituitary glands (McDonald et al. 1968a). Its specific activity on Lys-Ala-β-naphthylamide and Ala-Ala-Ala increased in parallel.

The purified enzyme hydrolyzed Lys-Ala-β-naphthylamide at 18, Lys-Ala-OMe at 27, and Ala-Ala-Ala at 44 μmoles min^{-1} mg^{-1} protein. As shown in Table 3, dipeptidyl aminopeptidase II did not act on fluorogenic substrates used for the assay of dipeptidyl aminopeptidase I, i.e., Gly-Arg-β-naphthylamide and Gly-Phe-β-naphthylamide. Amino acid β-naphthylamides were not hydrolyzed. Whereas dipeptidyl aminopeptidase II exhibited a fairly broad specificity on tripeptides (Table 4), no activity was detected on tetramethionine, trialanine methyl ester, tetraalanine, hexaalanine, or polyalanine. Thus, its specificity on alanine peptides was the reverse of that found for dipeptidyl aminopeptidase I (McDonald et al. 1969b). The action of dipeptidyl aminopeptidase II on other polypeptides and physiological substrates has, however, not yet been explored. Typical of a "dipeptidyl aminopeptidase," purified dipeptidyl aminopeptidase II cleaved NH_2-terminal dipeptides from dipeptide derivatives as well as from tripeptides. For example, Ser-Met was cleaved from Ser-Met-OMe and Ser-Met-Glu, but N^{α}-benzyloxycarbonyl-Ser-Met-Glu was not hydrolyzed.

Dipeptidyl aminopeptidase II had a pH 5.5 optimum on Lys-Ala-β-naphthylamide, and a 4.5 optimum on Ala-Ala-Ala. No metal, halide, or sulfhydryl dependence# ~~were~~ was detected, but a rather unique cation sensitivity was observed. The hydrolysis of Lys-Ala-β-naphthylamide was competitively inhibited by cations, and the percent inhibition was directly proportional to the square root of the atomic or molecular weight of the cation. Accordingly, Tris ion (K_i=0.32 mM) and puromycin (K_i=0.02 mM) were more inhibitory than sodium ion (K_i=1.8 mM). When rat tissues were surveyed for their relative activity on Lys-Ala-β-naphthylamide at pH 5.5 (Table 7), the activity measured in all the tissues was shown to be strongly inhibited by Tris ions. This finding was taken as presumptive evidence for the action of a common enzyme. p-Chloromercuriphenyl sulfonate and EDTA inhibitied neither the hydrolysis of

Ala-Ala-Ala nor Lys-Ala-β-naphthylamide.

3.2. Subcellular Localization

3.2.1. Biochemical studies

A lysosomal distribution was first indicated for dipeptidyl aminopeptidase II in the rat pituitary gland by conventional methods of differential and equilibrium-density centrifugation. The results of these studies, including those concerned with the latency and morphology of isopynic fractions, have already been reported (see section 2.7.1).

3.2.2. Electron microscopy

Lys-Ala-4-methoxy-β-naphthylamide was adopted as a specific substrate for the light and electron microscopic localization of dipeptidyl aminopeptidase II in the pituitary and thyroid of the rat. The histochemical reaction was conducted at pH 5.5. The technique was the same as already mentioned for the localization of dipeptidyl aminopeptidase I (see section 2.7.2).

Acid phosphatase is known to have a lysosomal distribution in cells of the anterior pituitary (Fig. 8). Similarly, dipeptidyl aminopeptidase II, which is discretely localized in the cells of the anterior pituitary gland (Fig. 9), was found to have a lysosomal distribution, as shown for a somatotrope (Fig. 10). These findings confirmed the results of an earlier biochemical study (McDonald et al. 1968b), that indicated a lysosomal distribution for dipeptidyl aminopeptidase II in the rat pituitary gland (see section 2.7.1).

Dipeptidyl aminopeptidase II was also found to have a lysosomal distribution in the rat thyroid, the tissue richest in this activity (see Table 7). As shown in Figs. 12 and 13, the enzyme activity was located

in lysosomes residing within the follicular cells. The lysosomes appeared to be more numerous near the pinocytosing (apical) surface bordering the colloid. The follicle shown in Fig. 14 reveals a discrete localization for dipeptidyl aminopeptidase II in all the cells of the secretory epithelium.

4. DIPEPTIDYL AMINOPEPTIDASE III

The cleavage of NH_2-terminal dipeptides from certain polypeptides can also be effected by a dipeptidyl aminopeptidase which has a hydrolytic optimum at pH 8 to 9. The peptidase was first detected in aqueous solutions obtained by extraction of anterior pituitary glands at pH 5.5 (Ellis and Nuenke 1967). Dipeptidyl aminopeptidase III was assayed fluorometrically by monitoring the release of β-naphthylamine from Arg-Arg-β-naphthylamide at pH 9. This substrate was not hydrolyzed by dipeptidyl aminopeptidase I, II, or IV. Dipeptidase and aminopolypeptidase (Ellis and Perry 1966) showed negligible activity on Arg-Arg-β-naphthylamide at pH 9.0. Arg-Arg-β-naphthylamide can therefore be employed to assay dipeptidyl aminopeptidase III with a high degree of specificity.

4.1. Specificities and Properties

Dipeptidyl aminopeptidase III, isolated from bovine pituitary glands, hydrolyzed Arg-Arg-β-naphthylamide optimally at pH 8.7, and for full activity required activation by sulfhydryl compounds. Activity was abolished by p-chloromercuriphenyl sulfonate or N-ethyl maleimide and was restored by treatment with sulfhydryl compounds. Although EDTA (1 mM) is highly inhibitory, the enzyme did not appear to have a metal requirement since the activity was restored either by complexing the EDTA with a variety of metallic ions or removing it by dialysis.

Analysis of the kinetics of Arg-Arg-β-naphthylamide hydrolysis in 0.0625M Tris-HCl at pH 9 and 37°C yielded a K_m of 0.0083 mM. The rate of hydrolysis was markedly decreased by substrate concentrations greater than 0.05 mM. Moreover, the hydrolysis product, Arg-Arg, at concentrations greater than 0.05 mM, completely inhibited the hydrolytic activity. Lys-Lys-β-naphthylamide, which was not hydrolyzed by dipeptidyl aminopeptidase III, was equally inhibitory.

Dipeptidyl aminopeptidase III did not hydrolyze the common monoamino acid β-naphthylamides. Of the dipeptide β-naphthylamides only Arg-Arg-β-naphthylamide was cleaved, and no hydrolysis was detected on the derivatives of Lys-Lys, Ala-Ala, Ser-Tyr, Leu-Ala, Gly-Pro, Gly-Arg, Gly-Phe, His-Ser or Ser-Met. At pH 7.5, Lys-Ala- and Ala-Ala-β-naphthylamide were hydrolyzed at less than 2% of the rate observed on Arg-Arg-β-naphthylamide at pH 9.0. This was ascribed to contamination with traces of dipeptidyl aminopeptidase II as indicated by different pH optima and an absence of activation by sulfhydryl compounds. The absence of action on the N^{α}-substituted derivative, benzyloxycarbonyl-Arg-Arg-β-naphthylamide, suggested that the enzymewas a dipeptidyl aminopeptidase.

A similar attack was observed on peptide substrates, except that the enzyme showed a broader substrate specificity on peptides than was first indicated by studies conducted on dipeptide β-naphthylamides. This was confirmed by examining the products generated from a series of alanine, lysine, and phenylalanine polypeptides. The substrates and the reaction products formed on complete hydrolysis were as follows:

$$Ala_4 \longrightarrow 2\ Ala_2 \qquad Lys_4 \longrightarrow 2\ Lys_2$$

$$Ala_5 \longrightarrow Ala_2 + Ala_3 \qquad Lys_5 \longrightarrow Lys_2 + Lys_3$$

$$Ala_6 \longrightarrow 3\ Ala_2 \qquad Phe_4 \longrightarrow 2\ Phe_2$$

On peptide substrates the enzyme had a pH optimum at about 8.0. Tetraglutamic acid and tetraglycine were not hydrolyzed. Since no hydrolysis occurred when the NH_2 terminus of tetraalanine was blocked by acetylation, it was concluded that the hydrolysis occurred sequentially from the NH_2 terminus. Since neither dipeptides nor tripeptides were cleaved, an additional requirement for the hydrolysis of simple peptides is that they contain at least four amino acid residues.

Certain polypeptides containing mixed amino acid residues were also cleaved with the release of dipeptides. Thus, Val-Leu-Ser-Glu-Gly was hydrolyzed to Val-Leu and Ser-Glu-Gly. The hydrolysis of angiotensin II with the sequential release of Asn-Arg and Val-Tyr from the NH_2 terminus is illustrated in Fig. 15. Further attack on the resulting tetrapeptide may have been prevented by the presence of the imide bond of proline as has been reported for dipeptidyl aminopeptidase I and several other peptidases. In contrast to the degradative effects of dipeptidyl aminopeptidase I on ACTH, dipeptidyl aminopeptidase III did not attack this peptide hormone detectably. Thus, while further studies are needed to define more precisely the specificity of dipeptidyl aminopeptidase III, it is apparent that, in comparison to dipeptidyl aminopeptidase I, its specificity is more restricted. However, the activity of dipeptidyl aminopeptidase III does appear to complement that of dipeptidyl aminopeptidase I, inasmuch as NH_2-terminal lysyl or arginyl residues blocked the action of dipeptidyl aminopeptidase I, but not that of dipeptidyl aminopeptidase III.

4.2. Subcellular Localization

Differential centrifugation of sucrose homogenates of bovine anterior pituitary glands revealed that 95% of the dipeptidyl aminopeptidase III was localized in the cell sap.

5. DIPEPTIDYL AMINOPEPTIDASE IV ("GLY-PRO NAPHTHYLAMIDASE")

Hopsu-Havu and his colleagues at the University of Turku, Finland, have described an intracellular enzyme that hydrolyzes Gly-Pro-β-naphthylamide. This enzyme, called "Gly-Pro naphthylamidase" by these workers, was first encountered in commercial preparations of porcine kidney acylase, and in rat liver extracts (Hopsu-Havu and Glenner 1966). The enzyme purified from rat liver (Hopsu-Havu and Sarimo 1967) and porcine kidney (Hopsu-Havu et al. 1968) showed comparable properties. Based on the substrate specificity reported for this enzyme, which has been confirmed and extended in our Laboratory, it seemed appropriate to classify this enzyme as a "dipeptidyl aminopeptidase." Herein, Gly-Pro naphthylamidase is referred to as dipeptidyl aminopeptidase IV.

5.1. Specificities and Properties

Hopsu-Havu and Sarimo (1967) showed that purified dipeptidyl aminopeptidase IV hydrolyzed Gly-Pro-β-naphthylamide at pH 7.8 to yield Gly-Pro and free β-naphthylamine. No free glycine or proline was detected. The β-naphthylamine reaction product was diazotized with fast garnet GBC and assayed colorimetrically. N^{α}-Benzyloxycarbonyl-Gly-Pro-β-naphthylamide was not hydrolyzed, and negligible rates were observed on amino acid β-naphthylamides. Proline β-naphthylamide was hydrolyzed at 0.2% the rate of Gly-Pro-β-naphthylamide, Leu-Leu-β-naphthylamide at 9% and Ala-Ala-β-naphthylamide at 7%.

Substrate specificity studies were also conducted in this Laboratory using a purified preparation of rat kidney dipeptidyl aminopeptidase IV that was generously provided by Dr. Hopsu-Havu. Initially, this

preparation had a specific activity on Gly-Pro-β-naphthylamide of 30 μmoles min^{-1} mg^{-1} protein at pH 8.0. As illustrated in Table 5, Gly-Pro-β-naphthylamide appeared to be the best fluorogenic substrate for the assay of the enzyme. Assays were conducted fluorometrically (McDonald et al. 1966a), and the initial rates of hydrolysis were compared. Rates were linear from the origin, which indicated a dipeptide cleavage rather than a stepwise breakdown of the substrate. Penultimate alanyl bonds were hydrolyzed slowly, and not at all when aspartic acid was NH_2-terminal. Terminal arginine or lysine residues did not prevent hydrolysis. The assay substrate for dipeptidyl aminopeptidase II (Lys-Ala-β-naphthylamide) was also hydrolyzed by dipeptidyl aminopeptidase IV. However, the two enzymes have widely separated pH optima, and this should preclude any interference in the assay of dipeptidyl aminopeptidase II in crude extracts. The assay substrates for dipeptidyl aminopeptidases I and III were not hydrolyzed. As judged by its action on the β-naphthylamide substrates, dipeptidyl aminopeptidase IV may prove to have a very selective degradative role in vivo. Its preference for proline bonds suggests that collagen fragments may be attacked.

Its aminopeptidase activity was also demonstrated on Gly-Pro-Ala and Gly-Pro-Gly-Gly (Hopsu-Havu and Sarimo 1967). The NH_2-terminal dipeptides were cleaved from these substrates, but not from N^{α}-benzyloxycarbonyl-Gly-Pro-Leu-Gly-Pro. These results have been confirmed in our Laboratory. In addition, we have found that Leu-Pro-Gly-Gly was readily split, whereas Gly-Pro-Pro was not attacked. It has also been reported that hemoglobin was not attacked (Hopsu-Havu and Glenner 1966).

Purified dipeptidyl aminopeptidase IV is unstable below pH 5.0. However, in crude extracts it tolerates 24 hours of incubation at pH 4.0 and 37°C.

In fact, this is the best way to solubilize this particle-bound enzyme for the purpose of purification (Hopsu-Havu and Sarimo 1967). We have found this enzyme to be present in autolyzed spleen and liver fractions used to purify lysosomal enzymes such as dipeptidyl aminopeptidase I, and cathepsins B and B'.

Dipeptidyl aminopeptidase IV from both rat liver (Hopsu-Havu and Sarimo 1967) and porcine kidney (Hopsu-Havu et al. 1968) had a pH optimum of 7.8 on Gly-Pro-β-naphthylamide. Whereas the rat liver enzyme had a K_m of 0.15 mM and a molecular weight of about 250,000, the porcine kidney enzyme had a K_m of 0.3 mM and a molecular weight of 270,000. However, these differences are of questionable significance.

The enzymatic activity of these preparations was not affected by sulfhydryl compounds, thiol reagents, organophosphorus compounds, or chelating agents. Certain heavy metals, i.e., Pb^{++}, Hg^{++}, and Zn^{++}, showed a strong inhibition between 10^{-4} and 10^{-5} M.

5.2. Subcellular Localization

Dipeptidyl aminopeptidase IV was located in microsomal fractions prepared from rat liver extracts by differential centrifugation. In fact, the liver enzyme was first purified (600 fold) from a microsomal fraction that was allowed to autolyze at pH 4.0 to solubilize the enzyme (Hopsu-Havu and Sarimo 1967).

Hopsu-Havu and Ekfors (1969) have used Gly-Pro-β-naphthylamide in a histochemical procedure to localize the enzyme in rat kidney, the organ richest in this activity (see Table 7). Their results showed a concentration of activity in the proximal convoluted tubuli, and somewhat less in the glomeruli. In the ovaries, fairly intense staining was observed in atrophic follicles and in the fibroblasts. In skin, the dermal fibroblasts showed

intense staining.

In our Laboratories, Gly-Pro-4-methoxy-β-naphthylamide has been adopted as a histochemical substrate for the localization of dipeptidyl aminopeptidase IV by both light and electron microscopic techniques (see section 2.7.2). However, at this time, only the results of light microscopy have been completed. In agreement with Hopsu-Havu and Ekfors (1969), the heaviest staining was observed in the luminal border of proximal cells.

6. DISCUSSION

The utilization of dipeptide β-naphthylamide substrates has facilitated the recognition and purification of a class of peptidases capable of cleaving unsubstituted NH_2-terminal dipeptides from a diversity of polypeptides. The specificity of this class of peptidases ranges from the rather general attack exhibited by dipeptidyl aminopeptidase I to the relatively high selectivity for prolyl bonds which is displayed by dipeptidyl aminopeptidase IV. The former enzyme possesses amidase and esterase activities and attacks peptides ranging in size from four residues as in tetraalanine to peptides as large as ACTH. Certain tripeptides are hydrolyzed but at relatively low rates. Dipeptidyl aminopeptidase II, on the other hand, seems restricted to the cleavage of tripeptides and dipeptide esters; its activity on large peptides has not yet been tested. Dipeptidyl aminopeptidase III does not hydrolyze peptides containing fewer than four residues, nor does it attack dipeptide amides and esters. Dipeptidyl aminopeptidase IV appears to be active principally on the penultimate prolyl bond of tripeptides and tetrapeptides, but data is not available regarding the activity on larger proline-containing peptides. The general properties of the presently known members of the class of dipeptidyl aminopeptidases are summarized in Table 6.

The dipeptidyl aminopeptidases are ubiquitously distributed in the tissues of the rat. However, it is evident that each enzyme displays a characteristic pattern of distribution among the various tissues (Table 7). Liver and spleen extracts are exceptionally rich in dipeptidyl aminopeptidase I whereas the thyroid and spleen contain the highest concentrations of dipeptidyl aminopeptidase II. Using Gly-Phe-NH_2 as substrate, Bouma and Gruber (1964) have observed essentially the same tissue distribution of dipeptidyl aminopeptidase I. In comparison with assays involving ammonia diffusion, the highly sensitive fluorometric method facilitated the dilution of tissue inhibitors, and eliminated complications that often arise from endogenous sources of ammonia. Although Ser-Tyr-β-naphthylamide was adopted for our tissue survey of dipeptidyl aminopeptidase I, the sensitivity of the assay could have been increased at least ten-fold through the use of Gly-Arg-β-naphthylamide as the substrate. However, it was suspected that cathepsin B' might also hydrolyze this substrate since it was reported to hydrolyze N^{α}-benzoyl-Arg-p-nitroanilide optimally at pH 5.5 (Otto 1967). We later found that our preparations of purified cathepsin B' (McDonald et al. 1970) hydrolyzed Gly-Arg-β-naphthylamide at 30% of the rate for N^{α}-benzoyl-Arg-β-naphthylamide. Since we now know that purified aminopeptidase I hydrolyzes Gly-Arg-β-naphthylamide 300 times faster than does purified cathepsin B', it therefore seems feasible to use Gly-Arg-β-naphthylamide for the assay of dipeptidyl aminopeptidase I in crude extracts.

The rat jejunum was found to contain a high level of dipeptidyl aminopeptidase I activity. Mucosal scrapings were sometimes found to approach spleen as a rich source of this activity. The jejunal activity in homogenates was inhibited to the same extent as the liver enzyme by an antiserum to the rat liver enzyme. The antiserum was effective at a 1:40,000 dilution in the fluorometric

assay system. Normal rabbit serum had no effect on the activity, and the antiserum did not inhibit dipeptidyl aminopeptidase II. The high concentration of dipeptidyl aminopeptidase I in the jejunal mucosa suggests that this peptidase may be significantly involved in the high cellular turnover that occurs in mucosal epithelium and possibly also in the terminal degradation of absorbed peptides.

Hopsu-Hava and Rintolą (1968) obtained the partially purified enzyme from kidney tissue and found it to parallel the specificity and activation requirements of dipeptidyl aminopeptidase I obtained from liver. The kidney, as already noted by Hopsu-Havu and Ekfors (1969) contains a remarkably high concentration of dipeptidyl aminopeptidase IV.

Changes in the levels of the dipeptidyl aminopeptidases of the anterior pituitary were observed by Vanha-Perttula (1969) following alteration of the functional status of the gland by castration or treatment with estradiol or testosterone. However, in the light of our present knowledge, it is not possible to evaluate the significance of changes in the tissue levels of these enzymes. While the patterns of distribution of the enzymes among different tissues is of value for purification purposes, they offer little insight into the role of the aminopeptidases in cellular metabolism.

Purified dipeptidyl aminopeptidase I has been reported to have no action on physiological substrates such as hemoglobin, serum albumin, egg albumin, ribonuclease, glucagon, and insulin (Planta and Gruber 1961, Metrione et al. 1966). These findings, together with the belief that dipeptidyl aminopeptidase I had an extremely narrow substrate specificity, led to the generally-accepted conclusion that the enzyme played an inconsequential role in intracellular protein catabolism. It now appears, however, that dipeptidyl aminopeptidase I may play a very significant role in tissue proteolysis

by working in conjunction with lysosomal proteases such as cathepsins B, B' and D. The demonstrated activities of dipeptidyl aminopeptidases I and II help to explain the findings of Coffey and de Duve (1968), who showed that globin substrates are reduced, predominantly, to amino acids and dipeptides by extracts of purified liver lysosomes; 40% of the globin residues were recovered as amino acids, and 30% as dipeptides. The carboxypeptidase activity of cathepsin A (Iodice 1967) may also contribute free amino acids to the pool. The production of relatively large amounts of dipeptides provides a raison d'etre for the soluble dipeptidases which have been reported to be localized in the non-particulate fraction from cells of the pituitary gland (Ellis and Perry 1966), brain (Marks, et al. 1968), kidney (Vanha-Perttula et al. 1966) and ascites carcinoma (Patterson et al. 1963). Since lysosomes are freely permeable to small organic molecules, it may be assumed that dipeptides resulting from the breakdown of proteins can diffuse into the cell sap where final conversion to free amino acids is accomplished by the soluble dipeptidases.

The lysosomal cathepsins which have been reasonably well characterized, namely, cathepsins A, B, B', D and dipeptidyl aminopeptidases I and II, encompass a broad spectrum of specificities which endow the lysosome with a discrete and powerful proteolytic system. One rather dramatic example of their digestive capacity is the process of "crinophagy" exhibited by pituitary lysosomes. Once a stressful stimulus has been terminated, hormone-bearing secretion granules accumulate within the secretory cells. Electron microscopic studies show that these "surplus" secretion granules fuse with lysosomes wherein they undergo extensive digestion (Smith and Farquhar 1966). Biochemical studies conducted by Ellis (1960) with highly purified pituitary proteinase I ("cathepsin D") have shown that 75 and 100%

of the biological activity of growth hormone and prolactin, respectively, are lost as a consequence of the cleavage of only seven peptide bonds in each of these hormones. Recent studies conducted in this Laboratory have revealed that growth hormone is extensively degraded by cathepsin B'. It is expected that pituitary dipeptidyl aminopeptidases I and II, together with the soluble dipeptidase and aminopolypeptidase of the pituitary (Ellis and Perry 1966), would continue this degradative process, and thereby return the constituent residues of these hormones to the amino acid pool of the gland.

The presence of additional extra-lysosomal peptidases in the cell sap and particulate elements suggests the existence of more specialized and, perhaps, alternate pathways for the degradation of peptides. For example, dipeptidyl aminopeptidase III of the cell sap may serve to further degrade oligopeptides released by an alkaline proteinase found in the secretion granules of anterior pituitary cells (Tesar et al. 1969). Specific, but as yet undefined, functions are also suggested by the presence in microsomes of dipeptidyl aminopeptidase IV (Hopsu-Havu and Sarimo 1967) and leucyl arylamidase (Patterson et al. 1963). Finally, the selectivity of the bonds cleaved in the conversion of proinsulin to insulin (Steiner et al. 1969) further suggests the existence of intracellular peptidases which possess highly specific functions.

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TABLE 1

NH_2-TERMINAL DIPEPTIDES LIBERATED FROM POLYPEPTIDE SUBSTRATES BY DIPEPTIDYL AMINOPEPTIDASE I (CATHEPSIN C)

NH_2-TERMINAL RESIDUE	COOH-TERMINAL RESIDUE: Ala	Arg	Asn	Asp	Cys	Cy SO_3H	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
Ala	+	+										+									
Arg	[−]	−										−				−					
Asn		+					+														
Asp	+	+							+						+		+			+	
Cys																					
Cy SO_3H									+												
Gln									+	+											
Glu	+	+						+		+		+									
Gly		[+]						+	+			+	+		+	[−]	+		[−]		
His												+			[+]		+				
Ile																					
Leu	[+]	+				+	+													+	+
Lys	[−]												−			−					
Met														+							
Phe		+													+					+	+
Pro		[+]																			
Ser		+						+		+			+	+						+	
Thr															+	−	+				
Trp									+					+							
Tyr												+									
Val			+			+		+				+								+	

A NEGATIVE (−) OR POSITIVE (+) RESULT SHOWN WITHIN A BOX (□) MEANS THAT THE ACTIVITY WAS TESTED WITH A DIPEPTIDE β-NAPHTHYLAMIDE SUBSTRATE ONLY.

Table 2

Specificity and kinetics of dipeptide β-naphthylamide hydrolysis by rat liver dipeptidyl aminopeptidase I (cathepsin C)

Substrate (-β-naphthylamide)	Activity[a]		Kinetics	
	Specific	Relative	$K_{m\ app}$ ± S.E.	k_{cat} ± S.E.
	μmoles min^{-1} mg^{-1} protein	%	mM	sec^{-1}
Gly-Arg	300	100	0.1 ± 0.006	1,300 ± 30
Cbz-Gly-Arg	0	0		
Ala-Arg (in secretin)	215	72		
Pro-Arg	164	55		
Ser-Met (in ACTH)	100	33	0.17 ± 0.03	510 ± 39
Ala-Ala (in Ala_4)	46	15	0.19 ± 0.01	248 ± 9
Glu-His (in ACTH)	33	11		
Ser-Tyr (in ACTH)	28	9		
Phe-Arg (in ACTH)	23	8		
Gly-Phe (in insulin B chain)	17	5.6	0.17 ± 0.01	79 ± 3
His-Ser (in glucagon)	16	5.3	0.022 ± 0.002	116 ± 6
Leu-Ala	10	3.4		
Lys-Ala	0	0		
Arg-Arg (in glucagon)	0	0		
Gly-Pro	0	0		

[a] Reaction mixtures contained 0.2 mM substrate; 10 mM 2-mercaptoethylamine hydrochloride; 10 mM cacodylic acid-NaOH, pH 6.0. Temperature 37°C. Reaction rates were measured by a direct fluorometric method (McDonald et al. 1966a).

TABLE 3

Rates of dipeptide β-naphthylamide hydrolysis at pH 5.5 by dipeptidyl aminopeptidase II from bovine anterior pituitary glands

Substrate (-β-naphthylamide)	Activity	
	Specific	Relative
	μmoles/min/mg	%
Lys-Ala	16	100
N^{α}, N^{ϵ} -dibenzyloxycarbonyl-Lys-Ala	0	0
Arg-Ala	4.8	30
Leu-Ala	1.6	10
Ala-Ala	0.8	5.0
Gly-Pro	0.7	4.5
Gly-Arg	0	0
Arg-Arg	0	0
Gly-Phe	0	0
Ser-Tyr	0	0
Ser-Met	0	0

No activity was detected on the β-naphythylamides of Asp-Ala, His-Ser, His-Phe, Lys-Lys, or Gly-Trp.

TABLE 4

Relative rates of tripeptide hydrolysis at pH 5.5 by dipeptidyl aminopeptidase II from bovine anterior pituitary glands

Substrate	Relative Activity
Ala-Ala-Ala	100
D-Ala-D-Ala-D-Ala	0
Met-Met-Ala	62
Met-Met-Met	33
Ser-Met-Glu	28
Ser-Met-Gln	23
Met-Gly-Met	9
Gly-Gly-Met	3
Phe-Gly-Gly	1
Phe-Phe-Phe	1
Tyr-Tyr-Tyr	1
Gly-Gly-Gly	0
Val-Val-Val	0

Hydrolysis was shown to involve the release of the NH_2-terminal dipeptide. Dipeptides liberated from homologous tripeptides were assumed to be from the NH_2 terminus.

TABLE 5

Rates of dipeptide β-naphthylamide hydrolysis at pH 8.0 by dipeptidyl aminopeptidase IV from rat kidney

Substrate (-β-naphthylamide)	Activity	
	Specific	Relative
	μmoles/min/mg	%
Gly-Pro	9.8	100
Pro	0.01	0.1
N^{α}-benzyloxycarbonyl-Gly-Pro	0	0
Leu-Ala	0.86	8.8
Arg-Ala	0.56	5.7
Lys-Ala	0.50	5.1
Ala-Ala	0.16	1.6
Asp-Ala	0	0
Gly-Phe	0	0
Gly-Arg	0	0
Arg-Arg	0	0

No activity was detected on the β-naphthylamides of Pro-Arg, Phe-Arg, Ala-Arg, N^{α}-benzoyl-Arg, His-Ser, His-Phe, Ser-Tyr, Ser-Met, or Gly-Trp.

TABLE 6

DIFFERENTIATION AND PROPERTIES OF DIPEPTIDYL AMINOPEPTIDASES

	DAP I[a]	DAP II[b]	DAP III[c]	DAP IV[d]
SPECIFIC ASSAY SUBSTRATE	Ser-Tyr-βNA Gly-Phe-βNA	Lys-Ala-βNA	Arg-Arg-βNA	Gly-Pro-βNA
PEPTIDE SUBSTRATES	TETRAPEPTIDES-POLYPEPTIDES (TRIPEPTIDES)	TRIPEPTIDES	TETRAPEPTIDES POLYPEPTIDES	TRI & TETRAPEPTIDES (POLYPEPTIDES?) PENULTIMATE PROLINE PREFERRED
pH OPTIMUM	5.0 - 6.0	4.5 - 5.5	8.0 - 9.0	7.6 - 7.8
K_m (37°, ASSAY SUBSTRATE)	0.17 mM (pH 6.0)	0.011 mM (pH 5.5)	0.008 mM (pH 9.0)	0.3 mM (pH 7.7)
MOLECULAR WEIGHT	ca. 210,000	ca. 130,000	ca. 80,000	ca. 250,000
ACTIVATORS	-SH , Cl^-	NONE	-SH	NONE
INHIBITORS	SULFHYDRYL REAGENTS OXYTOCIN	CATIONS: PUROMYCIN$^+$> TRIS$^+$> Na^+	EDTA	Pb^{++}; Hg^{++}, Zn^{++}
SUBCELLULAR LOCALIZATION	LYSOSOMAL	LYSOSOMAL	CELL SAP	MICROSOMAL
SOURCE OF ENZYME USED FOR CHARACTERIZATION	RAT LIVER BOVINE SPLEEN	BOVINE ANTERIOR PITUITARY	BOVINE ANTERIOR PITUITARY	PORCINE KIDNEY RAT LIVER

[a] DAP, dipeptidyl aminopeptidase; DAP I, CATHEPSIN C.
Fruton and Mycek 1956; Metrione et al. 1966; McDonald et al. 1966a, 1966b, 1969a, 1969b.

[b] McDonald et al. 1968a, 1968b.

[c] Ellis and Nuenke 1967.

[d] DAP IV, "Gly-Pro naphthylamidase."
Hopsu-Havu and Glenner 1966; Hopsu-Havu and Sarimo 1967; Hopsu-Havu et al. 1968.

TABLE 7

DISTRIBUTION OF DIPEPTIDYL AMINOPEPTIDASE (DAP) ACTIVITIES AMONG THE TISSUES OF THE RAT[a]

mμMOLES OF SUBSTRATE HYDROLYZED min^{-1} ml^{-1} 5% EXTRACT (50 mg TISSUE)
(MEAN ± S.E. BASED ON THREE RATS)

TISSUE	DAP I	DAP II	DAP III	DAP IV
LIVER	245 ± 24.0	22 ± 2.5	16 ± 1.5	33 ± 4.8
SPLEEN	190 ± 8.0	90 ± 9.7	84 ± 3.0	47 ± 1.8
LACRIMAL (EXORB.)	121 ± 8.7	57 ± 5.3	89 ± 1.0	71 ± 7.4
LUNG	102 ± 14	33 ± 2.5	68 ± 4.8	67 ± 13
JEJUNUM	94 ± 11	40 ± 9.0	66 ± 5.8	22 ± 7.8
PANCREAS	66 ± 3.7	14 ± 1.1	160 ± 14.0	9.6 ± 0.6
KIDNEY	55 ± 1.4	77 ± 3.0	59 ± 2.4	297.0 ± 11.6
THYROID	54 ± 7.0	112 ± 10.0	30 ± 4.9	6.2 ± 0.9
THYMUS	43 ± 0.3	53 ± 7.2	100 ± 2.8	22.0 ± 1.7
SEMINAL VES.	28 ± 4.6	17 ± 0.8	57 ± 10	2.9 ± 0.3
SUBMAXILLARY	20 ± 2.5	23 ± 4.3	93 ± 1.7	8.3 ± 0.8
COAGULATING GLAND	20 ± 6.2	20 ± 3.7	28 ± 0.2	2.9 ± 0.4
ADRENAL	12 ± 0.4	33 ± 1.0	31 ± 1.9	14.0 ± 1.5
TESTIS	8.4 ± 0.6	21 ± 0.5	41 ± 3.7	1.3 ± 0.03
PITUITARY	6.1 ± 0.6	68 ± 1.7	62 ± 1.1	2.1 ± 0.1
SKELETAL MUSCLE	3.1 ± 0.1	14 ± 1.1	12 ± 0.2	0.9 ± 0.02
VENTRAL PROSTATE	1.8 ± 0.12	12 ± 0.8	112 ± 10	4.9 ± 0.2
SERUM[b]	1.6 ± 0.13	4.4 ± 0.5	3 ± 0.2	11.0 ± 3.7
BRAIN (whole)	0.5 ± 0.04	14.0 ± 0.7	36 ± 2.6	1.03 ± 0.07

[a] Neutral extracts were obtained for assay from 5% (W/V) aqueous homogenates by removing cell debris at 10,000 × g for 30 min. Male, 400g Sprague-Dawley rats were used for all tissues.
DAP I on Ser-Tyr-βNA (pH 4); DAP II on Lys-Ala-βNA (pH 5·5); DAP III on Arg-Arg-βNA (pH 9);
DAP IV on Gly-Pro-βNA (pH 8)

[b] Rates per ml of undiluted serum

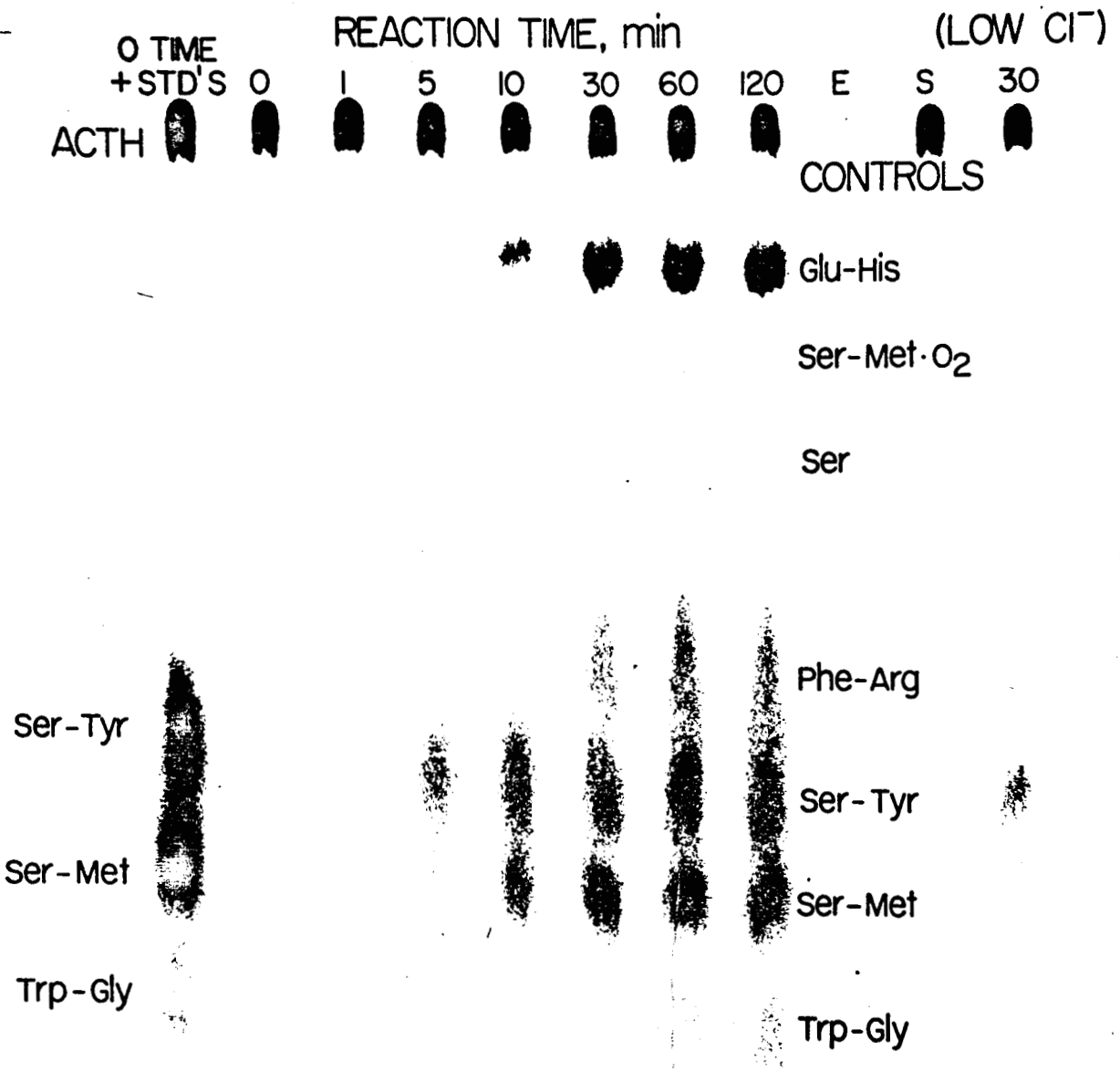
FIGURE LEGENDS

Fig. 1. Time course analysis of the degradation of β-corticotropin (ACTH) by rat liver dipeptidyl aminopeptidase I (cathepsin C). About 5×10^{-5} μmole enzyme and 1 μmole ACTH was incubated in a 2-mercaptoethanol-pyridine-HCl-acetic acid buffer, pH 5.0. The origin at the left contained a zero time aliquot (0.03 μmole ACTH) plus 0.03 μmole each of 3 dipeptide standards. Reaction times up to 120 min are represented. An enzyme control (E) and a substrate control (S) were incubated for 120 min. A 30-min reaction is shown that contained no added Cl^-. The products accumulated in the 120-min reaction mixture were identified as Glu-His, Ser-Met sulfone (Ser-Met-O_2), serine, Phe-Arg, Ser-Tyr, Ser-Met, and Trp-Gly. (From McDonald et al. 1969b.)

Fig. 2. Hydrolysis of polypeptide hormones by rat liver dipeptidyl aminopeptidase I (cathepsin C) at pH 5.0 to 5.5 in the presence of added Cl^- and -SH. The NH_2-terminal attack upon these hormones is illustrated and shows the particular peptide bonds that are cleaved during the consecutive removal of dipeptides. The vertical bars between glucagon and secretin indicate the points in the amino acid sequences where the constituent residues differ.

Fig. 3. Effect of concentration of various halide ions on the rate of Gly-Phe-β-naphthylamide hydrolysis by rat liver dipeptidyl aminopeptidase I (cathepsin C). The enzyme (40 ng of protein) and the appropriate halide salt were contained in a 4 ml, fluorometric assay system buffered at pH 6.0 with 10 mM mercaptoethanol-0.01 M cacodylic acid-NaOH buffer, pH 6.0. (From McDonald et al. 1969b.)

NH_2-TERMINUS OF ACTH Ser-Tyr | 1 Ser-Met | 2 Glu-His | 3 Phe-Arg | 4 Trp-Gly | 5 Lys-Pro-Val- ----------



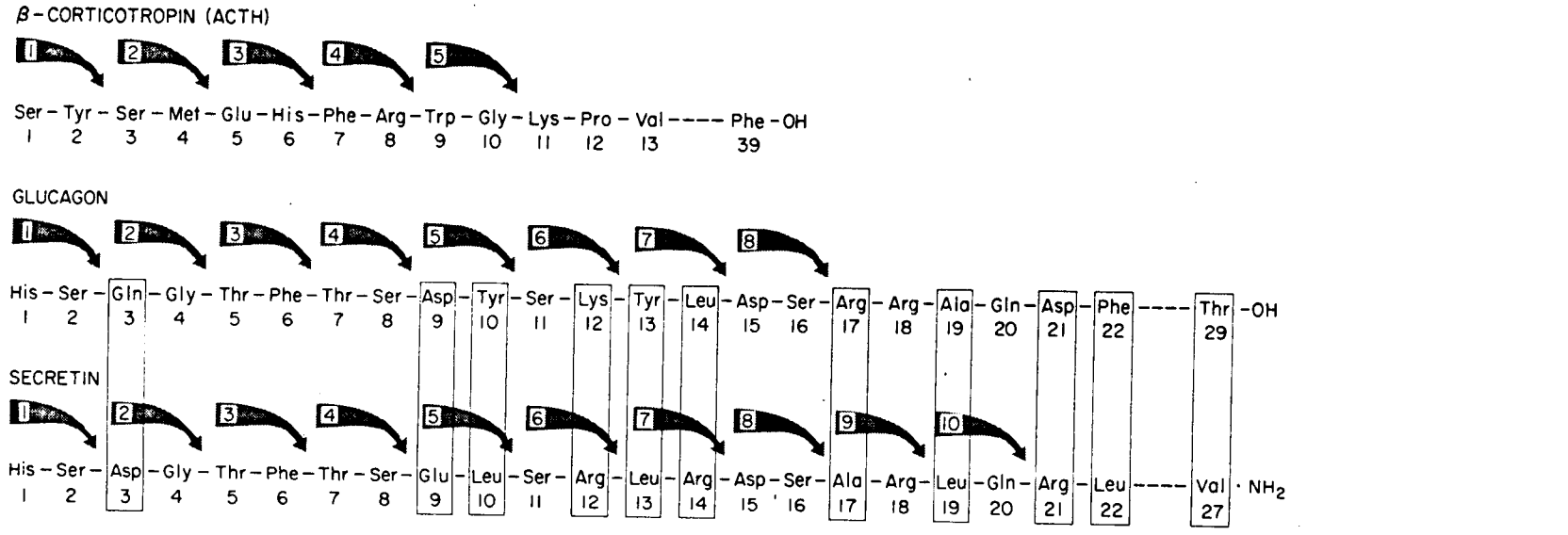
HYDROLYSIS OF ACTH BY CATHEPSIN C (WITH Cl⁻)

REACTION: CATHEPSIN C 10 μg/ml
ACTH 1 μ mole/ml } RATIO = 10
IN 16 mM HCL-0·8% PYRIDINE-15 mM MCE
TO pH 5·0 WITH ACETIC ACID

Figure 1

DEGRADATION OF PEPTIDE HORMONES BY DIPEPTIDYL AMINOPEPTIDASE I (CATHEPSIN C)*

(pH 5.0-5.5, Cl^- AND SH ESSENTIAL)



*THE SAME SPECIFICITY WAS OBSERVED WITH BOTH RAT LIVER AND BOVINE SPLEEN DAP I

Figure 2

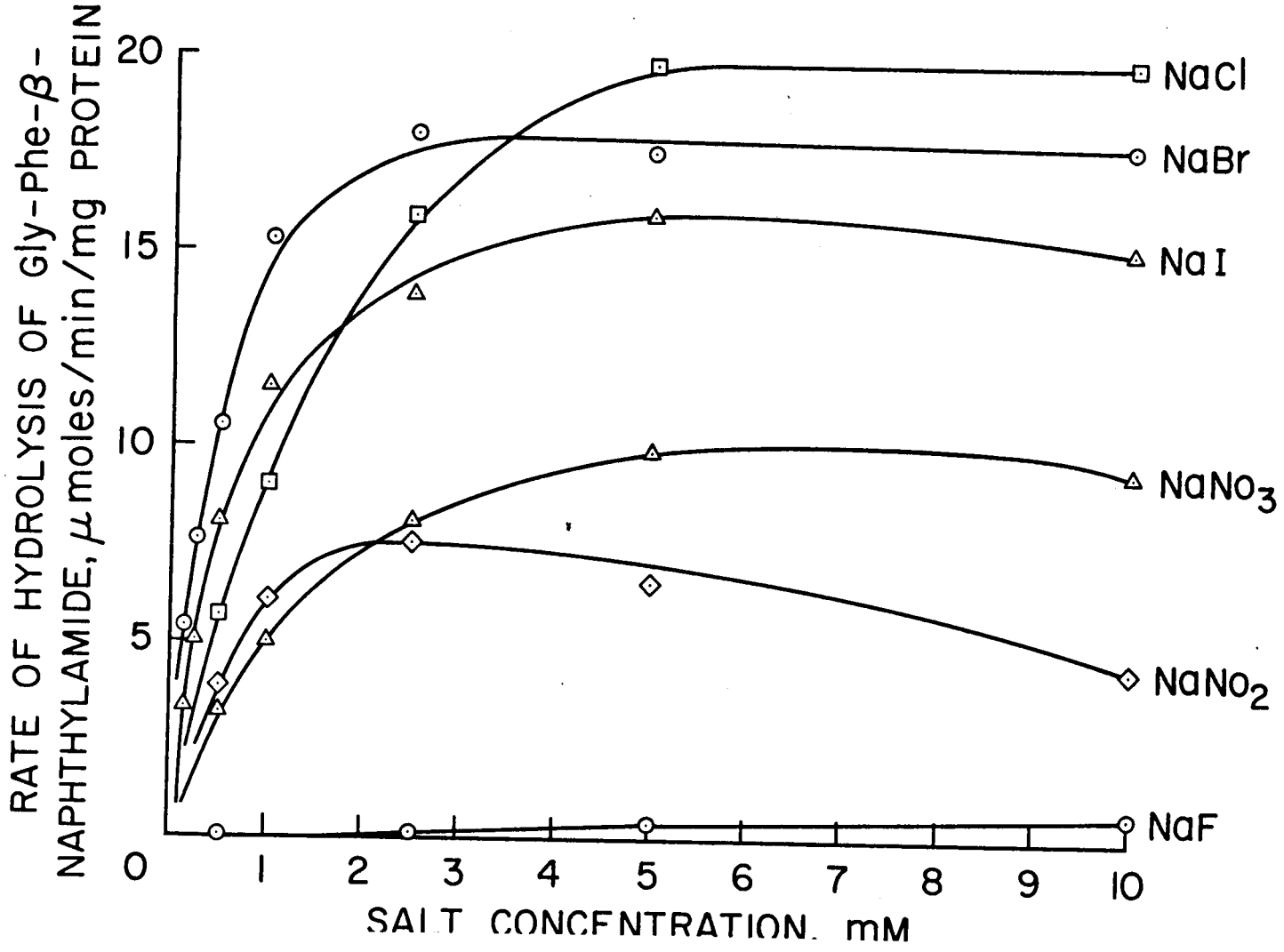

RATE OF HYDROLYSIS OF Gly-Phe-β-NAPHTHYLAMIDE, μ moles/min/mg PROTEIN
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
SALT CONCENTRATION, mM
NaCl
NaBr
NaI
NaNo3
NaNo2
NaF

Figure 3

PLATE I. Histochemical and immunochemical staining of liver lysosomes for dipeptidyl aminopeptidase I (cathepsin C).

Fig. 4. Lysosomal structures stained for dipeptidyl aminopeptidase I in the liver of a rat sacrificed 40 min after a single intraperitoneal injection of glucagon (50μg per 100g body weight). Note formation and staining of autophagic body (Ab) as well as lysosomes (L) of the dense-body type; (nucleus n). Pro-Arg-4-methoxy-β-naphthylamide as substrate and hexazotized pararosaniline were used in a simultaneous reaction at pH 5.5 for 20 min at 37°C. Mercaptoethylamine-HCl was used as a source of SH and Cl^-. X 21,000.

Fig. 5. Lysosomes (L) of the dense-body type stained for dipeptidyl aminopeptidase I in liver of a control rat. X 30,000.

Fig. 6. Immunochemical staining for dipeptidyl aminopeptidase I in an epoxy-embedded section (2μ) of liver using the peroxidase-labeled antibody procedure. (See Nakane and Pierce 1967.) X 1,000.

Fig. 7. Lysosome (L) staining for dipeptidyl aminopeptidase I by the peroxidase-labeled antibody procedure. Electron opacity was due to osmium chelated by oxidized 3,3'-diaminobenzidine. X 30,000.

All tissues (except liver used for immunochemical staining) were fixed in 1.5% distilled glutaraldehyde, pH 7.4, 310 mOsm. Tissues were perfused for 10 min and the fixation continued for 6 hours at 4°C. Non-frozen 20μ sections were cut, using an Oxford Vibratome, and stored at 4°C in 7% sucrose-0.1M cacodylic acid-NaOH buffer, pH 7.4, until used in cytochemical reactions. Liver used for immunochemical staining was similarly treated, except the tissue was fixed in 10% formalin in 0.05M phosphate buffer, pH 7.4. These techniques will be reported in detail elsewhere.

PLATE I

Fig. 4,5

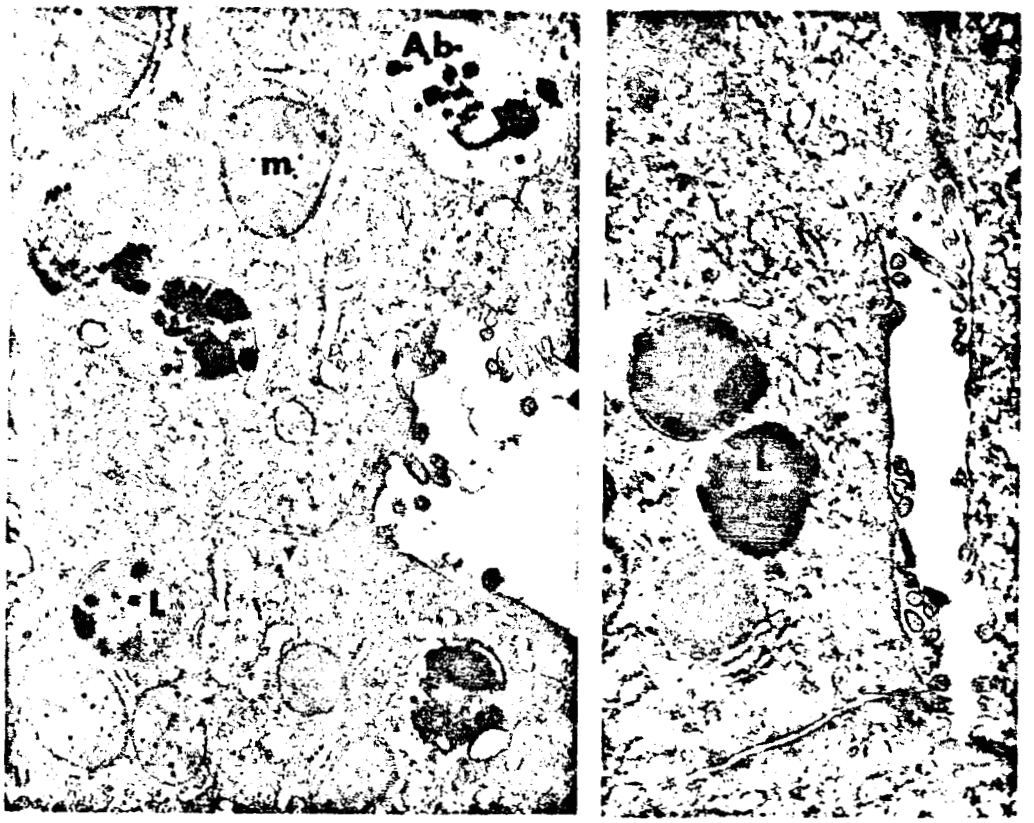


Fig. 6,7

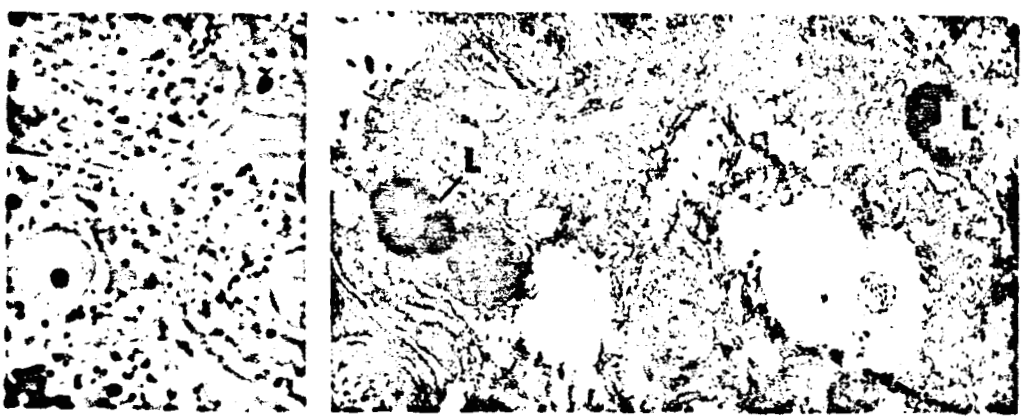


PLATE II. Pituitary lysosomes stained for acid phosphatase and dipeptidyl aminopeptidases I and II.

Fig. 8. Acid phosphatase staining with β-glycerophosphate lead salt technique. Localization of reaction product is in lysosomal structures of a gonadotrophic cell (multivescular body Mv; secretion granule Sg). (See Smith 1969a.) X 31,000.

Fig. 9. Epoxy-embedded section (2μ) of rat anterior pituitary gland stained for dipeptidyl aminopeptidase II with Lys-Ala-4-methoxy-β-naphthylamide as substrate and hexazotized pararosaniline in a simultaneous reaction at pH 5.5, 37°C, 4 min reaction time. X 800.

Fig. 10. Thin section adjacent to above 2μ section. Dipeptidyl aminopeptidase II staining of dense-body-type lysosomes (L) in a growth hormone secreting cell (secretion granule Sg; nucleus n). Electron opacity of reaction product due to chelated osmium. X 24,000.

Fig. 11. Lysosomes (L) stained for dipeptidyl aminopeptidase I in a gonadotrophic cell with Pro-Arg-4-methoxy-β-naphthylamide and hexazotized pararosaniline, 37°C, 35 min reaction time at pH 5.5. Staining of lysosomes for dipeptidyl aminopeptidase I is much less intense than for dipeptidyl aminopeptidase II. Biochemical studies (Table 7) show that the pituitary contains about 10 times more dipeptidyl aminopeptidase II. Note different degree of staining of three lysosomes. X 48,000.

PLATE II

Fig. 8,9

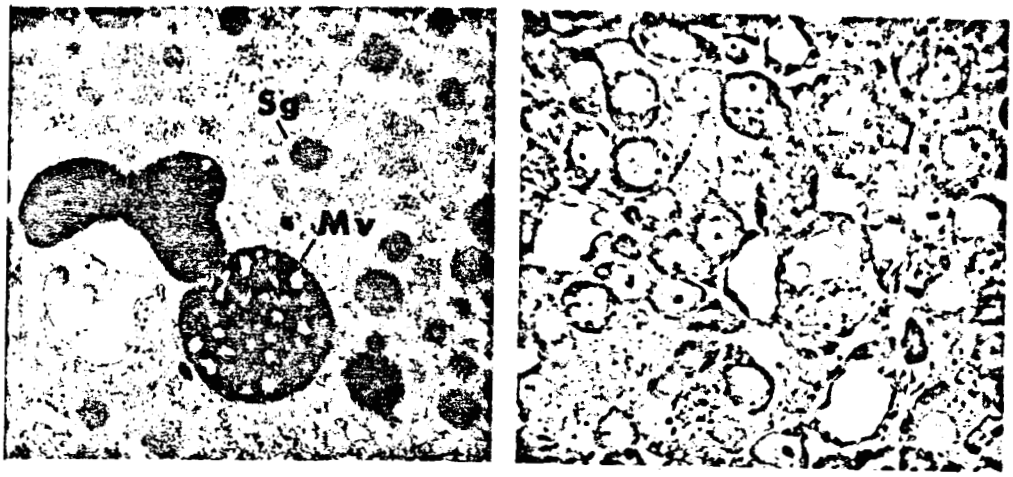


Fig. 10

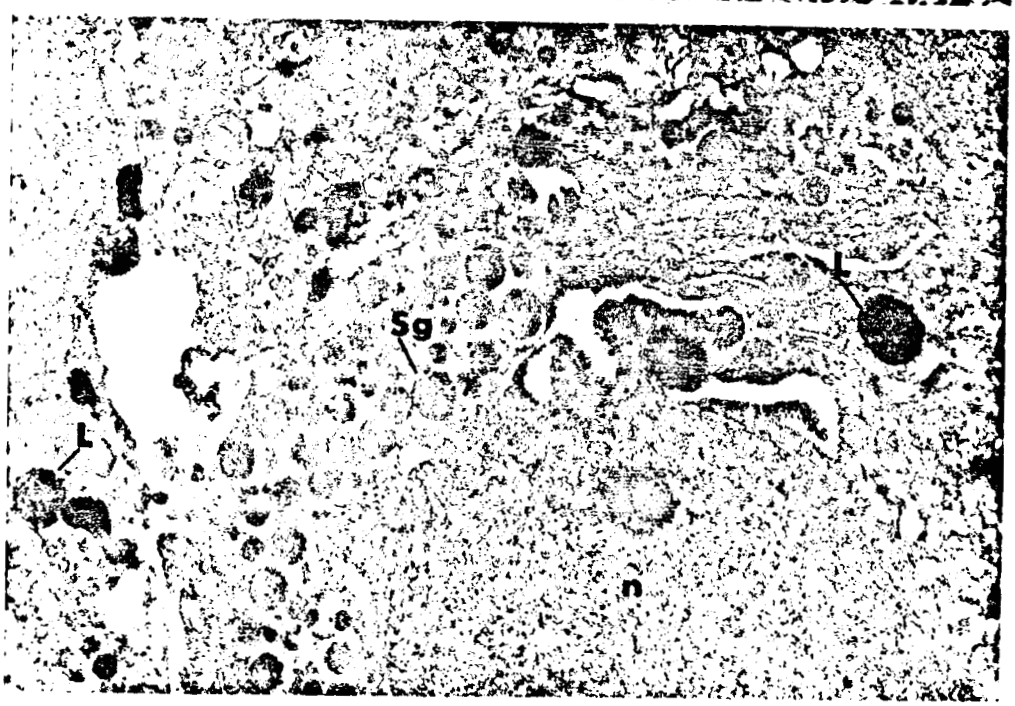


Fig. 11

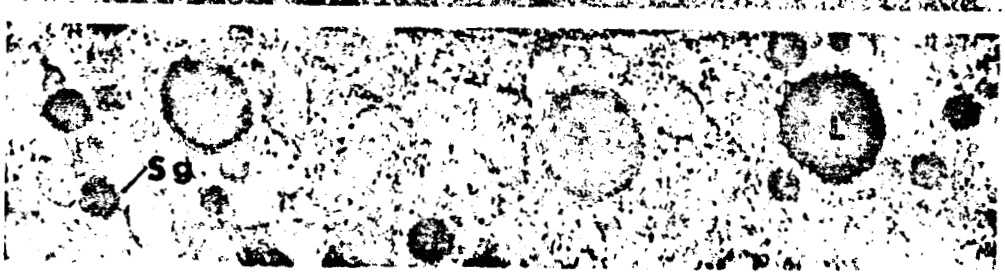


PLATE III. Thyroid lysosomes of the rat stained for dipeptidyl aminopeptidase II.

Fig. 12. Lysosomal staining for dipeptidyl aminopeptidase II with Lys-Ala-4-methoxy-β-naphthylamide as substrate and hexazotized pararosaniline in a simultaneous coupling reaction 37°C for 3 min at pH 5.5 (lysosome L; nucleus n; colloid Col). X 16,000.

Fig. 13. Apex of thyroid follicular cell with lysosomal staining for dipeptidyl aminopeptidase II. Note small lysosome (L) and adjoining colloid droplet. X 24,000.

Fig. 14. Epoxy-embedded thyroid section (2μ) stained for dipeptidyl aminopeptidase II. Note localized reaction product in epithelial cells of the follicle surrounding the colloid (Col). X 800.

PLATE III

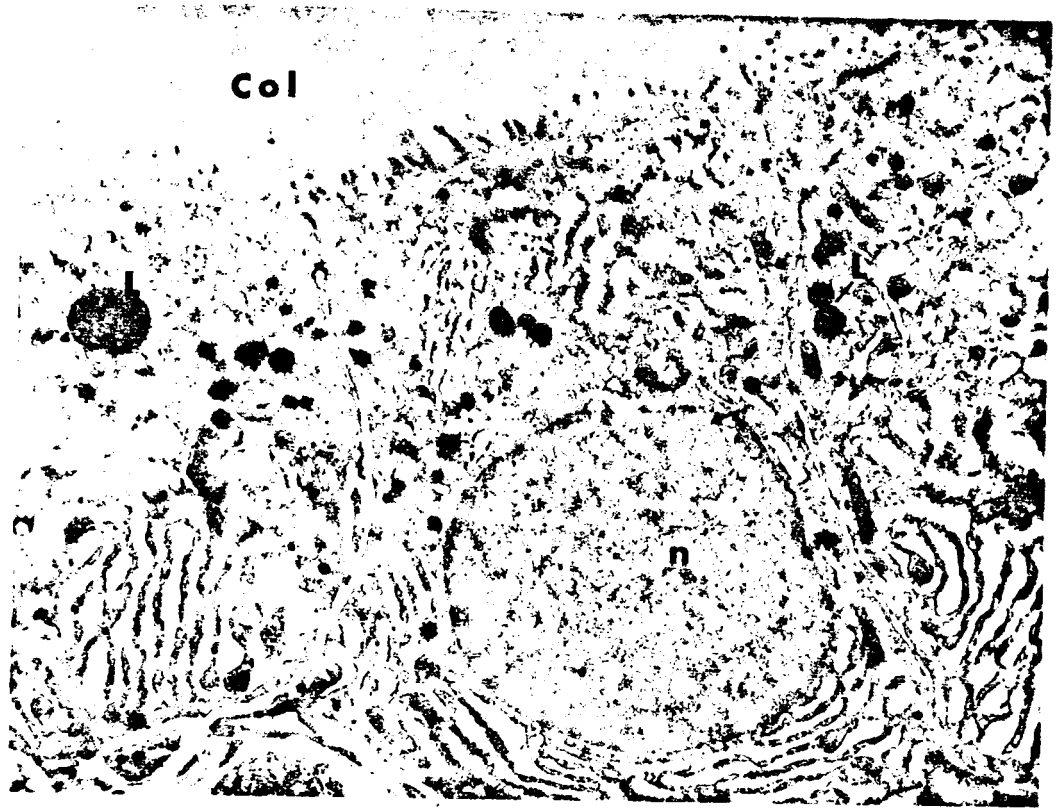


Fig. 12

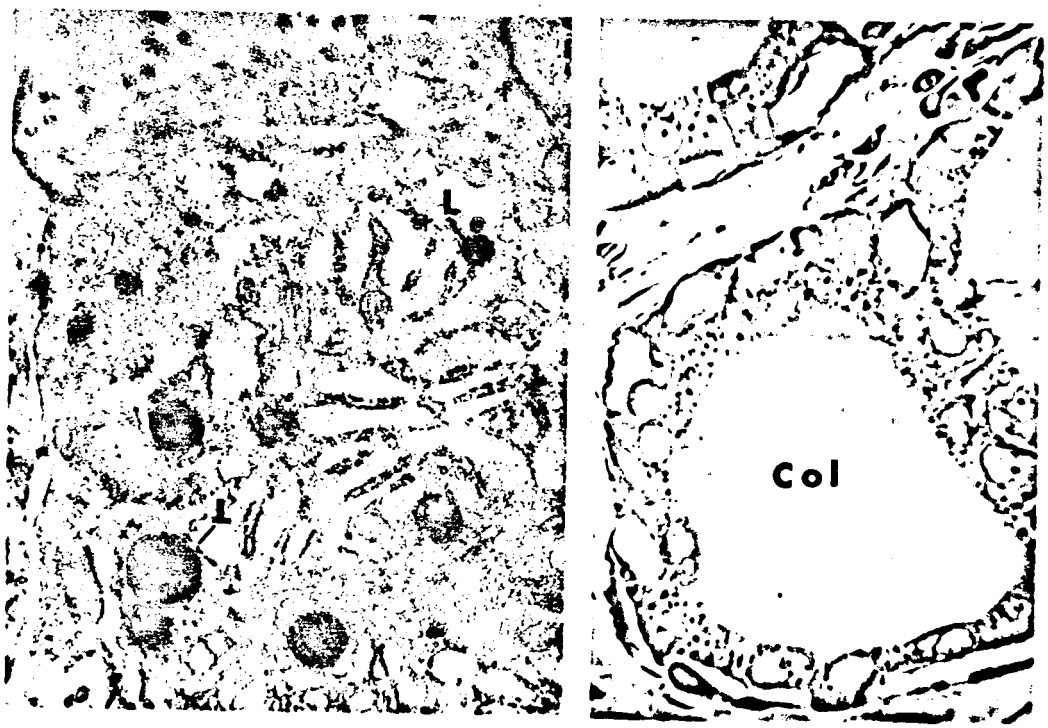


Fig.13,14

Fig. 15 Time course analysis of the hydrolysis of $Asp^1(NH_2)$-angiotensin II (CIBA) at pH 7.9 by purified dipeptidyl aminopeptidase III from bovine anterior pituitary glands. The reaction mixture contained 0.3 μmole of angiotensin II and 75 μg dipeptidyl aminopeptidase III in 0.1 ml of 1 mM dithiothreitol-0.1 M NH_4HCO_3, pH 7.9. The reaction mixture was incubated at 37°C, and, at the designated time intervals, 1 μl aliquots (equivalent to 3 nmoles of angiotensin) were applied to a thin layer of microcrystalline cellulose. Enzyme (E) and substrate (S) controls were incubated for 0 and 120 min. The minor component seen in the substrate is Asp^1-angiotensin II -- an equilibrium product formed during the synthesis of $Asp^1(NH_2)$-angiotensin II.

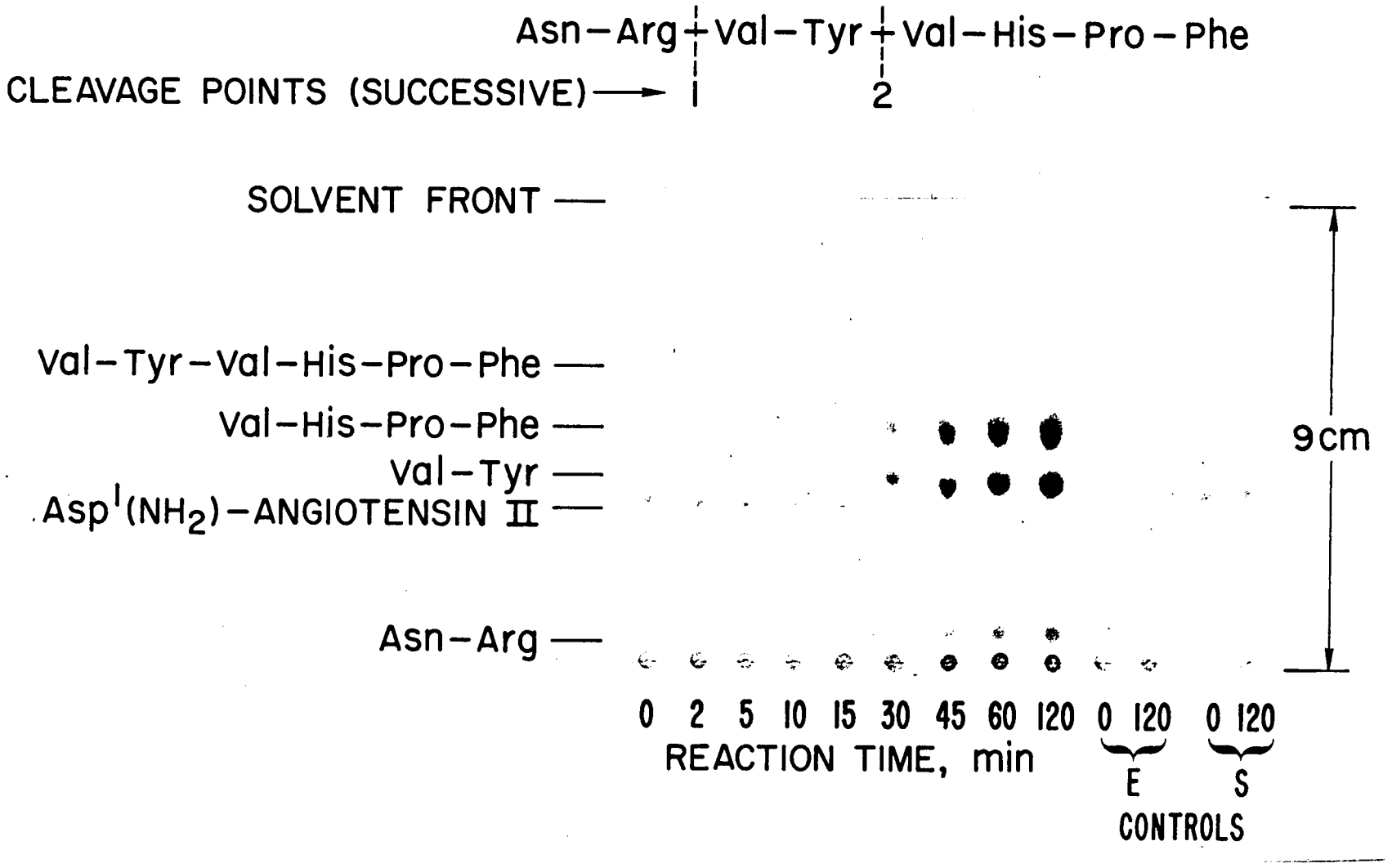

Asn–Arg + Val–Tyr + Val–His–Pro–Phe
CLEAVAGE POINTS (SUCCESSIVE) → 1 2
SOLVENT FRONT
Val–Tyr–Val–His–Pro–Phe
Val–His–Pro–Phe
Val–Tyr
Asp¹(NH2)–ANGIOTENSIN II
Asn–Arg
9cm
0 2 5 10 15 30 45 60 120 0 120 0 120
REACTION TIME, min
E
S
CONTROLS

Figure 15